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(54) **SOLUBILIZATION OF MSW WITH BLEND ENZYMES**

SOLUBILISIERUNG VON STÄDTISCHEN FESTSTOFFABFÄLLEN MIT MISCHUNGSENZYMEN

SOLUBILISATION DE DÉCHETS SOLIDES URBAINS À L'AIDE D'UN MÉLANGE D'ENZYMES

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Description**REFERENCE TO SEQUENCE LISTING**

5 [0001] This application contains a Sequence Listing in computer readable form.

FIELD OF THE INVENTION

10 [0002] The present invention relates to a method for solubilisation or hydrolysis of Municipal Solid Waste (MSW) with an enzyme blend, optionally for subsequent production of biogas and/or bioethanol.

BACKGROUND OF THE INVENTION

15 [0003] Municipal Solid Waste (MSW) is commonly also known as trash, garbage, refuse or rubbish. It consists of solid waste fractions that typically comes from municipalities and includes for instance waste from homes, schools, offices, hospitals, institutions etc. MSW is produced world-wide in very large quantities; thus in EU alone 2.44 million tons were generated in 2012 (Eurostat, 2014). The challenges of MSW production are many and may include collection, sorting, treatment, and disposal. Furthermore, well known environmental issues such as air and groundwater pollution from landfills is related to MSW. With an increasing world population entailing an increasing waste production, proper sustainable MSW management is a global challenge.

20 [0004] Although being environmental troublesome MSW also represents a large unexploited resource that may be used for energy production and recycling/recovery of scarce resources. Incineration is a technology that is widely used in some European countries, for instance Denmark, Sweden and Germany. The generation of energy is highly efficient but recovery of materials is limited. Furthermore, incineration of MSW results in a large production of slag (ash) which for some types of waste fractions can have environmental negative impacts (Idris and Saed 2002, Journal of Hazardous Materials B93 201-208). Capture of gas generated from anaerobic digestion of organic material at landfills is another way of generating energy from MSW but this technology also has a very low efficiency in materials recycling.

25 [0005] Integrated processes with concurrent energy production and recycling of materials are an attractive solution that has become a lot of attention lately. In such waste refineries, organic parts of MSW represent a resource that potentially can be used for bioenergy production in the form of for instance biogas (Hartmann and Ahning, 2006, Water Science & Technology Vol 53 No 8 p. 7-22). However, sorting of MSW in organic fractions for bioenergy production and plastic/metal fractions for material recovery is not easy due to the very inhomogeneous nature of MSW. As described by Jensen et al. (2012) (WO 2013/185777) presorting of MSW is typically costly, inefficient or impractical while source-sorting requires large infrastructure and operating expenses as well as active participation from the community from which the waste is collected.

30 [0006] Enzymatic treatment of MSW has lately been described and seems like a very interesting and innovative approach in the MSW management (Jensen et al. 2010, Waste Management 30, p. 2497-2503; Jensen et al. 2011, Biochem Biotechnol 165, p. 1799-1811; Tonini and Astrup 2012, Waste Management 32, p. 165-176). This technology is based on a liquefaction/solubilization step of organic degradable parts with hydrolytic enzymes and a subsequent separation of the MSW into a bioliquid and solids. The bioliquid can be used for biogas production while the solids can be further sorted and used for recycling or combusted according to the composition of the material. The technology has proven very robust at even high dry matter concentrations (35%) and has been demonstrated in pilot/demonstration facilities treating up to 1 ton of MSW/hour.

35 [0007] Tonini and Astrup (2012), evaluated the environmental sustainability - using life-cycle assessment - of four different waste refinery scenarios using this enzymatic liquefaction technology. Their assessment was based on a pilot-scale facility established at a Danish incinerator Amagerforbrænding in Copenhagen, Denmark. The different scenarios were compared to incineration. The authors concluded that "enzymatic refining of the waste with utilization of the products for energy recovery can represent a valuable alternative to incineration from both an energy and environmental point of view. This is the case if the downstream energy options for exploiting the solid and liquid fractions are co-combustion and anaerobic digestion for biogas production". The authors also concluded that cost savings of the waste refinery was related to a higher recovery of metals and energy. Furthermore, "improvement in the environmental as well as energy performance of the waste refinery itself was primarily related to the optimization of energy and enzymes consumption".

40 [0008] The use of cellulases (for instance Novozymes A/S Celluclast® 1.5 L, Novozymes A/S CELLIC® Ctec2 and Novozymes A/S CELLIC® Ctec3) for liquefaction of MSW with subsequent separation of unsorted waste into a bioliquid - used for biogas production - and into inorganic valuable products suitable for recycling has been clearly illustrated (WO2013/185777A1). However, with MSW being a complex substrate containing other organic components than cellulose (e.g. protein and lipids) it seems reasonable that the liquefaction process can be further improved by supplementing cellulases with other enzyme activities. Until now this theory has not been proven. An attempt was made Jensen *et al.*

2010 who tested a protease (Novozymes A/S Alcalase 2.5 L) and a α -amylase (Novozymes A/S Liquezyme® SC DC) as single enzymes and in combination with a cellulase (Novozymes A/S Celluclast® 1.5 L). "The cellulytic enzyme was the key catalyst in the liquefaction of degradable fractions both in terms of lowering viscosity and particle size distribution". No effect of the α -amylase and protease and interaction in between and with the cellulase was found. Nevertheless, it would be beneficial to provide an enzymes mix with a higher efficiency for liquefying MSW than the cellulases previously tested (Celluclast® 1.5L, CELLIC® Ctec2 and CELLIC® Ctec3). Such invention could contribute to a global change in MSW management practices and turn an environmental problem into a profitable and environmentally beneficial solution.

SUMMARY OF THE INVENTION

[0009] The present invention relates to an enzyme composition for solubilization of Municipal Solid Waste, the enzyme composition comprising a) an *Aspergillus fumigatus* cellobiohydrolase I; (b) an *Aspergillus fumigatus* cellobiohydrolase II; (c) an *Aspergillus fumigatus* beta-glucosidase; and (d) a *Penicillium* sp. GH61 polypeptide having cellulolytic enhancing activity and enzymes selected from (i) a protease; (ii) a lipase and (iii) a beta-glucanase, wherein: the protease (i) is selected from SEQ ID NO: 1 or a protease having at least 90% sequence identity to SEQ ID NO: 1; the lipase (ii) is selected from SEQ ID NO: 2 or a lipase having at least 90% sequence identity to SEQ ID NO: 2; the beta-glucanase is selected from SEQ ID NO: 4 or a beta-glucanase having at least 90% sequence identity to SEQ ID NO: 4.

[0010] As apparent from the findings disclosed herein, it was surprisingly found that a synergistic effect in solubilization of the MSW was obtained when adding blends of different enzymes to a cellulolytic background composition (27.1%), compared to the individual contributions of e.g. components *B.a protease*, *T.l pholip* and *A.a BG* which was up to 5%, 8.5% and 8.2% respectively.

BRIEF DESCRIPTION OF DRAWINGS

[0011]

Figure 1 shows solubilization of model waste in free fall experiments at two different dry matter concentrations. The figure shows the distribution of dry matter liquid (grey bars) and solids fractions (white bars).

Figure 2 shows a plot of data from dose response experiments with blend enzymes and CBC and model waste. The figure illustrates the dry matter found in the liquid fraction (TS solubilization) at different enzymes concentration. Figure 3 shows a plot of data from dose response experiments with blend enzymes and CBC and model waste. The figure illustrates sum of glucose and xylose (g/l) at different enzymes concentration.

Figure 4 shows a graph illustrating the effect of removing either *B.a protease*, *T.l pholip* or *A.a BG* components from the optimized blend. The figure illustrates the dry matter found in the liquid fraction (TS solubilization).

Figure 5 shows a data table from dose response experiments with blend enzymes and CBC and model waste. The table illustrates the amount of glucose, xylose and lactic acid at different enzyme concentrations.

DEFINITIONS

[0012] Acetylxyylan esterase: The term "acetylxyylan esterase" means a carboxylesterase (EC 3.1.1.72) that catalyzes the hydrolysis of acetyl groups from polymeric xylan, acetylated xylose, acetylated glucose, alpha-naphthyl acetate, and p-nitrophenyl acetate. Acetylxyylan esterase activity can be determined using 0.5 mM p-nitrophenylacetate as substrate in 50 mM sodium acetate pH 5.0 containing 0.01% TWEEN™ 20 (polyoxyethylene sorbitan monolaurate). One unit of acetylxyylan esterase is defined as the amount of enzyme capable of releasing 1 μ mole of p-nitrophenolate anion per minute at pH 5, 25°C.

[0013] Allelic variant: The term "allelic variant" means any of two or more alternative forms of a gene occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and may result in polymorphism within populations. Gene mutations can be silent (no change in the encoded polypeptide) or may encode polypeptides having altered amino acid sequences. An allelic variant of a polypeptide is a polypeptide encoded by an allelic variant of a gene.

[0014] Alpha-L-arabinofuranosidase: The term "alpha-L-arabinofuranosidase" means an alpha-L-arabinofuranoside arabinofuranohydrolase (EC 3.2.1.55) that catalyzes the hydrolysis of terminal non-reducing alpha-L-arabinofuranoside residues in alpha-L-arabinosides. The enzyme acts on alpha-L-arabinofuranosides, alpha-L-arabinans containing (1,3)- and/or (1,5)-linkages, arabinoxylans, and arabinogalactans. Alpha-L-arabinofuranosidase is also known as arabinosidase, alpha-arabinosidase, alpha-L-arabinosidase, alpha-arabinofuranosidase, polysaccharide alpha-L-arabinofuranosidase, alpha-L-arabinofuranoside hydrolase, L-arabinosidase, or alpha-L-arabinanase. Alpha-L-arabinofuranosidase activity can be determined using 5 mg of medium viscosity wheat arabinoxylan (Megazyme International Ireland, Ltd., Bray, Co. Wicklow, Ireland) per ml of 100 mM sodium acetate pH 5 in a total volume of 200 μ l for 30 minutes at 40°C

followed by arabinose analysis by AMINEX® HPX-87H column chromatography (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

[0015] Alpha-glucuronidase: The term "alpha-glucuronidase" means an alpha-D-glucosiduronate glucuronohydrolase (EC 3.2.1.139) that catalyzes the hydrolysis of an alpha-D-glucuronoside to D-glucuronate and an alcohol. Alpha-glucuronidase activity can be determined according to de Vries, 1998, J. Bacteriol. 180: 243-249. One unit of alpha-glucuronidase equals the amount of enzyme capable of releasing 1 μ mole of glucuronic or 4-O-methylglucuronic acid per minute at pH 5, 40°C.

[0016] Auxiliary Activity 9 polypeptide: The term "Auxiliary Activity 9 polypeptide" or "AA9 polypeptide" means a polypeptide classified as a lytic polysaccharide monooxygenase (Quinlan et al., 2011, Proc. Natl. Acad. Sci. USA 208: 15079-15084; Phillips et al., 2011, ACS Chem. Biol. 6: 1399-1406; Lin et al., 2012, Structure 20: 1051-1061). AA9 polypeptides were formerly classified into the glycoside hydrolase Family 61 (GH61) according to Henrissat, 1991, Biochem. J. 280: 309-316, and Henrissat and Bairoch, 1996, Biochem. J. 316: 695-696.

[0017] AA9 polypeptides enhance the hydrolysis of a cellulosic material by an enzyme having cellulolytic activity. Cellulolytic enhancing activity can be determined by measuring the increase in reducing sugars or the increase of the total of cellobiose and glucose from the hydrolysis of a cellulosic material by cellulolytic enzyme under the following conditions: 1-50 mg of total protein/g of cellulose in pretreated corn stover (PCS), wherein total protein is comprised of 50-99.5% w/w cellulolytic enzyme protein and 0.5-50% w/w protein of an AA9 polypeptide for 1-7 days at a suitable temperature, such as 40°C-80°C, e.g., 40°C, 45°C, 50°C, 55°C, 60°C, 65°C, 70°C, 75°C, or 80°C and a suitable pH, such as 4-9, e.g., 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, or 9.0, compared to a control hydrolysis with equal total protein loading without cellulolytic enhancing activity (1-50 mg of cellulolytic protein/g of cellulose in PCS).

[0018] AA9 polypeptide enhancing activity can be determined using a mixture of CELLUCLAST® 1.5L (Novozymes A/S, Bagsværd, Denmark) and beta-glucosidase as the source of the cellulolytic activity, wherein the beta-glucosidase is present at a weight of at least 2-5% protein of the cellulase protein loading. In one aspect, the beta-glucosidase is an *Aspergillus oryzae* beta-glucosidase (e.g., recombinantly produced in *Aspergillus oryzae* according to WO 02/095014). In another aspect, the beta-glucosidase is an *Aspergillus fumigatus* beta-glucosidase (e.g., recombinantly produced in *Aspergillus oryzae* as described in WO 02/095014).

[0019] AA9 polypeptide enhancing activity can also be determined by incubating an AA9 polypeptide with 0.5% phosphoric acid swollen cellulose (PASC), 100 mM sodium acetate pH 5, 1 mM MnSO₄, 0.1% gallic acid, 0.025 mg/ml of *Aspergillus fumigatus* beta-glucosidase, and 0.01% TRITON® X-100 (4-(1,1,3,3-tetramethylbutyl)phenyl-polyethylene glycol) for 24-96 hours at 40°C followed by determination of the glucose released from the PASC.

[0020] AA9 polypeptide enhancing activity can also be determined according to WO 2013/028928 for high temperature compositions.

[0021] AA9 polypeptides enhance the hydrolysis of a cellulosic material catalyzed by enzyme having cellulolytic activity by reducing the amount of cellulolytic enzyme required to reach the same degree of hydrolysis preferably at least 1.01-fold, e.g., at least 1.05-fold, at least 1.10-fold, at least 1.25-fold, at least 1.5-fold, at least 2-fold, at least 3-fold, at least 4-fold, at least 5-fold, at least 10-fold, or at least 20-fold.

[0022] The AA9 polypeptide can also be used in the presence of a soluble activating divalent metal cation according to WO 2008/151043 or WO 2012/122518, e.g., manganese or copper.

[0023] The AA9 polypeptide can be used in the presence of a dioxy compound, a bicyclic compound, a heterocyclic compound, a nitrogen-containing compound, a quinone compound, a sulfur-containing compound, or a liquor obtained from a pretreated cellulosic or hemicellulosic material such as pretreated corn stover (WO 2012/021394, WO 2012/021395, WO 2012/021396, WO 2012/021399, WO 2012/021400, WO 2012/021401, WO 2012/021408, and WO 2012/021410).

[0024] Beta-glucanase: The term "beta-glucanase" means any type of endo-beta-glucanase that hydrolyzes (1,3)- or (1,4)-linkages in beta-D-glucans (E.C. 3.2.1.73) (E.C.3.2.1.6).

[0025] Beta-glucosidase: The term "beta-glucosidase" means a beta-D-glucoside glucohydrolase (E.C. 3.2.1.21) that catalyzes the hydrolysis of terminal non-reducing beta-D-glucose residues with the release of beta-D-glucose. Beta-glucosidase activity can be determined using p-nitrophenyl-beta-D-glucopyranoside as substrate according to the procedure of Venturi et al., 2002, J. Basic Microbiol. 42: 55-66. One unit of beta-glucosidase is defined as 1.0 μ mole of p-nitrophenolate anion produced per minute at 25°C, pH 4.8 from 1 mM p-nitrophenyl-beta-D-glucopyranoside as substrate in 50 mM sodium citrate containing 0.01% TWEEN® 20.

[0026] Beta-xylosidase: The term "beta-xylosidase" means a beta-D-xyloside xylohydrolase (E.C. 3.2.1.37) that catalyzes the exo-hydrolysis of short beta (1 $\rightarrow$ 4)-xylooligosaccharides to remove successive D-xylose residues from non-reducing termini. Beta-xylosidase activity can be determined using 1 mM p-nitrophenyl-beta-D-xyloside as substrate in 100 mM sodium citrate containing 0.01% TWEEN® 20 at pH 5, 40°C. One unit of beta-xylosidase is defined as 1.0 μ mole of p-nitrophenolate anion produced per minute at 40°C, pH 5 from 1 mM p-nitrophenyl-beta-D-xyloside in 100 mM sodium citrate containing 0.01% TWEEN® 20.

[0027] Binding domain: The term "binding domain" e.g., "cellulose binding domain" means the region of an enzyme

that mediates binding of the enzyme to amorphous regions of a cellulose substrate. The cellulose binding domain (CBD) is typically found either at the N-terminal or at the C-terminal extremity of an enzyme.

[0028] Catalytic domain: The term "catalytic domain" means the region of an enzyme containing the catalytic machinery of the enzyme.

[0029] Carbohydrate binding module: The term "carbohydrate binding module" means a domain within a carbohydrate-active enzyme that provides carbohydrate-binding activity (Boraston et al., 2004, Biochem. J. 383: 769-781). A majority of known carbohydrate binding modules (CBMs) are contiguous amino acid sequences with a discrete fold. The carbohydrate binding module (CBM) is typically found either at the N-terminal or at the C-terminal extremity of an enzyme. Some CBMs are known to have specificity for cellulose.

[0030] Catalase: The term "catalase" means a hydrogen-peroxide:hydrogen-peroxide oxidoreductase (EC 1.11.1.6) that catalyzes the conversion of $2 \text{H}_2\text{O}_2$ to $\text{O}_2 + 2 \text{H}_2\text{O}$. For purposes of the present invention, catalase activity is determined according to U.S. Patent No. 5,646,025. One unit of catalase activity equals the amount of enzyme that catalyzes the oxidation of 1 μmole of hydrogen peroxide under the assay conditions.

[0031] Catalytic domain: The term "catalytic domain" means the region of an enzyme containing the catalytic machinery of the enzyme.

[0032] Cellobiohydrolase: The term "cellobiohydrolase" means a 1,4-beta-D-glucan cellobiohydrolase (E.C. 3.2.1.91 and E.C. 3.2.1.176) that catalyzes the hydrolysis of 1,4-beta-D-glucosidic linkages in cellulose, cellobiosaccharides, or any beta-1,4-linked glucose containing polymer, releasing cellobiose from the reducing end (cellobiohydrolase I) or non-reducing end (cellobiohydrolase II) of the chain (Teeri, 1997, Trends in Biotechnology 15: 160-167; Teeri et al., 1998, Biochem. Soc. Trans. 26: 173-178). Cellobiohydrolase activity can be determined according to the procedures described by Lever et al., 1972, Anal. Biochem. 47: 273-279; van Tilbeurgh et al., 1982, FEBS Letters 149: 152-156; van Tilbeurgh and Claeyssens, 1985, FEBS Letters 187: 283-288; and Tomme et al., 1988, Eur. J. Biochem. 170: 575-581.

Cellulolytic background composition (CBC) or Cellulolytic Enzyme Blend:

[0033] The term "Cellulolytic background composition" or "CBC" means an enzyme composition comprising a mixture of two or more cellulolytic enzymes. In one embodiment the CBC comprises two or more cellulolytic enzymes selected from: i) an *Aspergillus fumigatus* cellobiohydrolase I; (ii) an *Aspergillus fumigatus* cellobiohydrolase II; (iii) an *Aspergillus fumigatus* beta-glucosidase or variant thereof; and (iv) a *Penicillium sp.* GH61 polypeptide having cellulolytic enhancing activity; or homologs thereof. The CBC may further comprise one or more enzymes selected from: (a) an *Aspergillus fumigatus* xylanase or homolog thereof, (b) an *Aspergillus fumigatus* beta-xylosidase or homolog thereof; or (c) a combination of (a) and (b) (as described in further detail in WO 2013/028928). The CBC may be any CBC described in WO 2013/028928. In one embodiment, the CBC is CELLIC® Ctec3 obtainable from Novozymes A/S (Bagsværd, Denmark).

[0034] Cellulolytic enzyme or cellulase: The term "cellulolytic enzyme" or "cellulase" means one or more (e.g., several) enzymes that hydrolyze a cellulosic material. Such enzymes include endoglucanase(s), cellobiohydrolase(s), beta-glucosidase(s), or combinations thereof. The two basic approaches for measuring cellulolytic enzyme activity include: (1) measuring the total cellulolytic enzyme activity, and (2) measuring the individual cellulolytic enzyme activities (endoglucanases, cellobiohydrolases, and beta-glucosidases) as reviewed in Zhang et al., 2006, Biotechnology Advances 24: 452-481. Total cellulolytic enzyme activity can be measured using insoluble substrates, including Whatman No. 1 filter paper, microcrystalline cellulose, bacterial cellulose, algal cellulose, cotton, pretreated lignocellulose, etc. The most common total cellulolytic activity assay is the filter paper assay using Whatman No. 1 filter paper as the substrate. The assay was established by the International Union of Pure and Applied Chemistry (IUPAC) (Ghose, 1987, Pure Appl. Chem. 59: 257-68).

[0035] Cellulolytic enzyme activity can be determined by measuring the increase in production/release of sugars during hydrolysis of a cellulosic material by cellulolytic enzyme(s) under the following conditions: 1-50 mg of cellulolytic enzyme protein/g of cellulose in pretreated corn stover (PCS) (or other pretreated cellulosic material) for 3-7 days at a suitable temperature such as 40°C-80°C, e.g., 40°C, 45°C, 50°C, 55°C, 60°C, 65°C, 70°C, 75°C, or 80°C, and a suitable pH, such as 4-9, e.g., 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, or 9.0, compared to a control hydrolysis without addition of cellulolytic enzyme protein. Typical conditions are 1 ml reactions, washed or unwashed PCS, 5% insoluble solids (dry weight), 50 mM sodium acetate pH 5, 1 mM MnSO_4 , 50°C, 55°C, or 60°C, 72 hours, sugar analysis by AMINEX® HPX-87H column chromatography (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

[0036] Cellulosic material: The term "cellulosic material" means any material containing cellulose.

[0037] In an embodiment, the cellulosic material is agricultural residue, herbaceous material (including energy crops), municipal solid waste, pulp and paper mill residue, waste paper, or wood (including forestry residue).

[0038] cDNA: The term "cDNA" means a DNA molecule that can be prepared by reverse transcription from a mature, spliced, mRNA molecule obtained from a eukaryotic or prokaryotic cell. cDNA lacks intron sequences that may be present in the corresponding genomic DNA. The initial, primary RNA transcript is a precursor to mRNA that is processed through a series of steps, including splicing, before appearing as mature spliced mRNA.

[0039] Coding sequence: The term "coding sequence" means a polynucleotide, which directly specifies the amino acid sequence of a polypeptide. The boundaries of the coding sequence are generally determined by an open reading frame, which begins with a start codon such as ATG, GTG, or TTG and ends with a stop codon such as TAA, TAG, or TGA. The coding sequence may be a genomic DNA, cDNA, synthetic DNA, or a combination thereof.

[0040] Control sequences: The term "control sequences" means nucleic acid sequences necessary for expression of a polynucleotide encoding a mature polypeptide of the present invention. Each control sequence may be native (*i.e.*, from the same gene) or foreign (*i.e.*, from a different gene) to the polynucleotide encoding the polypeptide or native or foreign to each other. Such control sequences include, but are not limited to, a leader, polyadenylation sequence, propeptide sequence, promoter, signal peptide sequence, and transcription terminator. At a minimum, the control sequences include a promoter, and transcriptional and translational stop signals. The control sequences may be provided with linkers for the purpose of introducing specific restriction sites facilitating ligation of the control sequences with the coding region of the polynucleotide encoding a polypeptide.

[0041] Dissolved Oxygen Saturation Level: The saturation level of oxygen is determined at the standard partial pressure (0.21 atmosphere) of oxygen. The saturation level at the standard partial pressure of oxygen is dependent on the temperature and solute concentrations. In an embodiment where the temperature during hydrolysis is 50°C, the saturation level would typically be in the range of 5-5.5 mg oxygen per kg slurry, depending on the solute concentrations. Hence, a concentration of dissolved oxygen of 0.5 to 10% of the saturation level at 50°C corresponds to an amount of dissolved oxygen in a range from 0.025 ppm ($0.5 \times 5/100$) to 0.55 ppm ($10 \times 5.5/100$), such as, *e.g.*, 0.05 to 0.165 ppm, and a concentration of dissolved oxygen of 10-70% of the saturation level at 50°C corresponds to an amount of dissolved oxygen in a range from 0.50 ppm ($10 \times 5/100$) to 3.85 ppm ($70 \times 5.5/100$), such as, *e.g.*, 1 to 2 ppm. In an embodiment, oxygen is added in an amount in the range of 0.5 to 5 ppm, such as 0.5 to 4.5 ppm, 0.5 to 4 ppm, 0.5 to 3.5 ppm, 0.5 to 3 ppm, 0.5 to 2.5 ppm, or 0.5 to 2 ppm.

[0042] Endoglucanase: The term "endoglucanase" means a 4-(1,3;1,4)-beta-D-glucan 4-glucanohydrolase (E.C. 3.2.1.4) that catalyzes endohydrolysis of 1,4-beta-D-glycosidic linkages in cellulose, cellulose derivatives (such as carboxymethyl cellulose and hydroxyethyl cellulose), lichenin, beta-1,4 bonds in mixed beta-1,3-1,4 glucans such as cereal beta-D-glucans or xyloglucans, and other plant material containing cellulosic components. Endoglucanase activity can be determined by measuring reduction in substrate viscosity or increase in reducing ends determined by a reducing sugar assay (Zhang et al., 2006, *Biotechnology Advances* 24: 452-481). Endoglucanase activity can also be determined using carboxymethyl cellulose (CMC) as substrate according to the procedure of Ghose, 1987, *Pure and Appl. Chem.* 59: 257-268, at pH 5, 40°C.

[0043] Expression: The term "expression" includes any step involved in the production of a polypeptide including, but not limited to, transcription, post-transcriptional modification, translation, post-translational modification, and secretion.

[0044] Expression vector: The term "expression vector" means a linear or circular DNA molecule that comprises a polynucleotide encoding a polypeptide and is operably linked to control sequences that provide for its expression.

[0045] Feruloyl esterase: The term "feruloyl esterase" means a 4-hydroxy-3-methoxycinnamoyl-sugar hydrolase (EC 3.1.1.73) that catalyzes the hydrolysis of 4-hydroxy-3-methoxycinnamoyl (feruloyl) groups from esterified sugar, which is usually arabinose in natural biomass substrates, to produce ferulate (4-hydroxy-3-methoxycinnamate). Feruloyl esterase (FAE) is also known as ferulic acid esterase, hydroxycinnamoyl esterase, FAE-III, cinnamoyl ester hydrolase, FAEA, cinnAE, FAE-I, or FAE-II. Feruloyl esterase activity can be determined using 0.5 mM p-nitrophenylferulate as substrate in 50 mM sodium acetate pH 5.0. One unit of feruloyl esterase equals the amount of enzyme capable of releasing 1 μ mole of p-nitrophenolate anion per minute at pH 5, 25°C.

[0046] Fragment: The term "fragment" means a polypeptide or a catalytic or binding domain having one or more (*e.g.*, several) amino acids absent from the amino and/or carboxyl terminus of a mature polypeptide or domain; wherein the fragment has enzymatic or substrate binding activity.

[0047] Hemicellulolytic enzyme or hemicellulase: The term "hemicellulolytic enzyme" or "hemicellulase" means one or more (*e.g.*, several) enzymes that hydrolyze a hemicellulosic material. See, for example, Shallom and Shoham, 2003, *Current Opinion In Microbiology* 6(3): 219-228). Hemicellulases are key components in the degradation of plant biomass. Examples of hemicellulases include, but are not limited to, an acetylmannan esterase, an acetylxylnase, an arabinanase, an arabinofuranosidase, a coumaric acid esterase, a feruloyl esterase, a galactosidase, a glucuronidase, a glucuronoyl esterase, a mannanase, a mannosidase, a xylanase, and a xylosidase. The substrates for these enzymes, hemicelluloses, are a heterogeneous group of branched and linear polysaccharides that are bound via hydrogen bonds to the cellulose microfibrils in the plant cell wall, crosslinking them into a robust network. Hemicelluloses are also covalently attached to lignin, forming together with cellulose a highly complex structure. The variable structure and organization of hemicelluloses require the concerted action of many enzymes for its complete degradation. The catalytic modules of hemicellulases are either glycoside hydrolases (GHs) that hydrolyze glycosidic bonds, or carbohydrate esterases (CEs), which hydrolyze ester linkages of acetate or ferulic acid side groups. These catalytic modules, based on homology of their primary sequence, can be assigned into GH and CE families. Some families, with an overall similar fold, can be

further grouped into clans, marked alphabetically (e.g., GH-A). A most informative and updated classification of these and other carbohydrate active enzymes is available in the Carbohydrate-Active Enzymes (CAZy) database. Hemicellulolytic enzyme activities can be measured according to Ghose and Bisaria, 1987, Pure & Appl. Chem. 59: 1739-1752, at a suitable temperature such as 40°C-80°C, e.g., 40°C, 45°C, 50°C, 55°C, 60°C, 65°C, 70°C, 75°C, or 80°C, and a suitable pH such as 4-9, e.g., 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, or 9.0.

[0048] Hemicellulosic material: The term "hemicellulosic material" means any material comprising hemicelluloses.

[0049] Host cell: The term "host cell" means any cell type that is susceptible to transformation, transfection, transduction, or the like with a nucleic acid construct or expression vector comprising a polynucleotide of the present invention. The term "host cell" encompasses any progeny of a parent cell that is not identical to the parent cell due to mutations that occur during replication.

[0050] Isolated: The term "isolated" means a substance in a form or environment that does not occur in nature. Non-limiting examples of isolated substances include (1) any non-naturally occurring substance, (2) any substance including, but not limited to, any enzyme, variant, nucleic acid, protein, peptide or cofactor, that is at least partially removed from one or more or all of the naturally occurring constituents with which it is associated in nature; (3) any substance modified by the hand of man relative to that substance found in nature; or (4) any substance modified by increasing the amount of the substance relative to other components with which it is naturally associated (e.g., recombinant production in a host cell; multiple copies of a gene encoding the substance; and use of a stronger promoter than the promoter naturally associated with the gene encoding the substance).

[0051] Lipase: The term "lipase" means any enzyme that catalyzes the hydrolysis of lipids and/or having hydrolytic activity in class EC 3.1.1.- as defined by Enzyme Nomenclature. Particular useful is triacyl glycerol lipases (E.C.3.1.1.3) and phospholipase A1 (EC 3.1.1.32) and phospholipase A2 (E.C.3.1.1.4), but also other phospholipases (E.C.3.1.1.5), (E.C.3.1.4.4), (E.C.3.1.4.11), (E.C.3.1.4.50), (E.C.3.1.4.54).

[0052] Mannanases: In the context of the present invention a "mannanase" is a beta-mannanase and defined as an enzyme belonging to EC 3.2.1.78 or E.C.3.2.1.25. Mannanases have been identified in several *Bacillus* organisms. For example, Talbot et al., Appl. Environ. Microbiol., Vol.56, No. 11, pp. 3505-3510 (1990) describes a beta-mannanase derived from *Bacillus stearothermophilus* having an optimum pH of 5.5-7.5. Mendoza et al., World J. Microbiol. Biotech., Vol. 10, No. 5, pp. 551-555 (1994) describes a beta-mannanase derived from *Bacillus subtilis* having an optimum activity at pH 5.0 and 55°C. JP-03047076 discloses a beta-mannanase derived from *Bacillus sp.*, having an optimum pH of 8-10. JP-63056289 describes the production of an alkaline, thermostable beta-mannanase. JP-08051975 discloses alkaline beta-mannanases from alkalophilic *Bacillus sp.* AM-001. A purified mannanase from *Bacillus amyloliquefaciens* is disclosed in WO 97/11164. WO 94/25576 discloses an enzyme from *Aspergillus aculeatus*, CBS 101.43, exhibiting mannanase activity and WO 93/24622 discloses a mannanase isolated from *Trichoderma reesei*.

[0053] Mature polypeptide: The term "mature polypeptide" means a polypeptide in its final form following translation and any post-translational modifications, such as N-terminal processing, C-terminal truncation, glycosylation, phosphorylation, etc.

[0054] Mature polypeptide coding sequence: The term "mature polypeptide coding sequence" means a polynucleotide that encodes a mature polypeptide having enzymatic activity.

[0055] Municipal Solid Waste (MSW): The term "municipal solid waste" or "MSW" is intended to mean solid waste fractions that is typically available in municipalities (cities, towns, villages). MSW can be a combination of plant materials (fruit, vegetables, grains, corn etc), animal materials (meats etc.), cellulosic material (paper, cardboard, diapers, textile etc.), glass, plastic, metal. MSW includes the following but is not limited to any one or more of the following: waste collected from homes, schools, hospitals, offices, business, industries such as restaurants and food processing industries. MSW can potentially have been treated by shredding or pulping devices.

[0056] Nucleic acid construct: The term "nucleic acid construct" means a nucleic acid molecule, either single- or double-stranded, which is isolated from a naturally occurring gene or is modified to contain segments of nucleic acids in a manner that would not otherwise exist in nature or which is synthetic, which comprises one or more control sequences.

[0057] Operably linked: The term "operably linked" means a configuration in which a control sequence is placed at an appropriate position relative to the coding sequence of a polynucleotide such that the control sequence directs expression of the coding sequence.

[0058] Pretreated municipal solid waste material: The term "pretreated municipal solid waste material" means a municipal solid waste material derived from biomass by treatment with heat and dilute sulfuric acid, alkaline pretreatment, neutral pretreatment, or any pretreatment known in the art.

[0059] Protease: The term "protease" means any protease or proteolytic enzyme suitable for use under neutral or acidic conditions. Suitable proteases include those of animal, vegetable or microbial origin. Chemically or genetically modified mutants are included. Suitable proteases includes metallo endoprotease that hydrolyzes internal peptide bonds (E.C. 3.4.24.28), serine endoprotease that hydrolyzes internal peptide bonds (E.C.3.4.23.23), endoprotease that hydrolyzes peptide bonds at the carboxy side of lysine and arginine residues E.C.3.4.21.4), aminopeptidase (E.C. 3.4.11.1) and exopeptidase that liberates amino acids by hydrolysis of the N-terminal peptide bond (E.C. 3.4.11.1).

[0060] Sequence identity: The relatedness between two amino acid sequences or between two nucleotide sequences is described by the parameter "sequence identity".

[0061] For purposes of the present invention, the sequence identity between two amino acid sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, J. Mol. Biol. 48: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice et al., 2000, Trends Genet. 16: 276-277), preferably version 5.0.0 or later. The parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix. The output of Needle labeled "longest identity" (obtained using the -nobrief option) is used as the percent identity and is calculated as follows:

$$(\text{Identical Residues} \times 100) / (\text{Length of Alignment} - \text{Total Number of Gaps in Alignment})$$

[0062] For purposes of the present invention, the sequence identity between two deoxyribonucleotide sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *supra*) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice et al., 2000, *supra*), preferably version 5.0.0 or later. The parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EDNAFULL (EMBOSS version of NCBI NUC4.4) substitution matrix. The output of Needle labeled "longest identity" (obtained using the -nobrief option) is used as the percent identity and is calculated as follows:

$$(\text{Identical Deoxyribonucleotides} \times 100) / (\text{Length of Alignment} - \text{Total Number of Gaps in Alignment})$$

[0063] Solubilization: The term "solubilization" means enzymatic treatment of a substrate. In present disclosure, the terms "hydrolyzation", "liquefaction", "saccharification" and "solubilization" may be used interchangeably.

[0064] Variant: The term "variant" means a polypeptide comprising an alteration, *i.e.*, a substitution, insertion, and/or deletion, at one or more (*e.g.*, several) positions. A substitution means replacement of the amino acid occupying a position with a different amino acid; a deletion means removal of the amino acid occupying a position; and an insertion means adding an amino acid adjacent to and immediately following the amino acid occupying a position.

[0065] Xylan-containing material: The term "xylan-containing material" means any material comprising a plant cell wall polysaccharide containing a backbone of beta-(1-4)-linked xylose residues. Xylans of terrestrial plants are heteropolymers possessing a beta-(1-4)-D-xylopyranose backbone, which is branched by short carbohydrate chains. They comprise D-glucuronic acid or its 4-O-methyl ether, L-arabinose, and/or various oligosaccharides, composed of D-xylose, L-arabinose, D- or L-galactose, and D-glucose. Xylan-type polysaccharides can be divided into homoxylans and heteroxylans, which include glucuronoxylans, (arabino)glucuronoxylans, (glucurono)arabinoxylans, arabinoxylans, and complex heteroxylans. See, for example, Ebringerova et al., 2005, Adv. Polym. Sci. 186: 1-67.

[0066] In the processes of the present invention, any material containing xylan may be used. In a preferred aspect, the xylan-containing material is lignocellulose.

[0067] Xylan degrading activity or xylanolytic activity: The term "xylan degrading activity" or "xylanolytic activity" means a biological activity that hydrolyzes xylan-containing material. The two basic approaches for measuring xylanolytic activity include: (1) measuring the total xylanolytic activity, and (2) measuring the individual xylanolytic activities (*e.g.*, endoxylanases, beta-xylosidases, arabinofuranosidases, alpha-glucuronidases, acetylxylan esterases, feruloyl esterases, and alpha-glucuronyl esterases). Recent progress in assays of xylanolytic enzymes was summarized in several publications including Biely and Puchard, 2006, Journal of the Science of Food and Agriculture 86(11): 1636-1647; Spanikova and Biely, 2006, FEBS Letters 580(19): 4597-4601; Herrmann et al., 1997, Biochemical Journal 321: 375-381.

[0068] Total xylan degrading activity can be measured by determining the reducing sugars formed from various types of xylan, including, for example, oat spelt, beechwood, and larchwood xylans, or by photometric determination of dyed xylan fragments released from various covalently dyed xylans. A common total xylanolytic activity assay is based on production of reducing sugars from polymeric 4-O-methyl glucuronoxylan as described in Bailey et al., 1992, Interlaboratory testing of methods for assay of xylanase activity, Journal of Biotechnology 23(3): 257-270. Xylanase activity can also be determined with 0.2% AZCL-arabinoxylan as substrate in 0.01% TRITON® X-100 and 200 mM sodium phosphate pH 6 at 37°C. One unit of xylanase activity is defined as 1.0 μmole of azurine produced per minute at 37°C, pH 6 from 0.2% AZCL-arabinoxylan as substrate in 200 mM sodium phosphate pH 6.

[0069] Xylan degrading activity can be determined by measuring the increase in hydrolysis of birchwood xylan (Sigma Chemical Co., Inc., St. Louis, MO, USA) by xylan-degrading enzyme(s) under the following typical conditions: 1 ml reactions, 5 mg/ml substrate (total solids), 5 mg of xylanolytic protein/g of substrate, 50 mM sodium acetate pH 5, 50°C, 24 hours, sugar analysis using p-hydroxybenzoic acid hydrazide (PHBAH) assay as described by Lever, 1972, Anal.

Biochem. 47: 273-279.

[0070] Xylanase: The term "xylanase" means a 1,4-beta-D-xylan-xylohydrolase (E.C. 3.2.1.8) that catalyzes the endohydrolysis of 1,4-beta-D-xylosidic linkages in xylans. Xylanase activity can be determined with 0.2% AZCL-arabinoxylan as substrate in 0.01% TRITON® X-100 and 200 mM sodium phosphate pH 6 at 37°C. One unit of xylanase activity is defined as 1.0 μmole of azurine produced per minute at 37°C, pH 6 from 0.2% AZCL-arabinoxylan as substrate in 200 mM sodium phosphate pH 6.

[0071] Reference to "about" a value or parameter herein includes aspects that are directed to that value or parameter *per se*. For example, description referring to "about X" includes the aspect "X".

[0072] As used herein and in the appended claims, the singular forms "a," "or," and "the" include plural referents unless the context clearly dictates otherwise. It is understood that the aspects of the invention described herein include "consisting" and/or "consisting essentially of" aspects.

[0073] Unless defined otherwise or clearly indicated by context, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

DETAILED DESCRIPTION OF THE INVENTION

[0074] In some aspects of the disclosure enzymatic solubilization of MSW is carried out together with natural occurring microorganisms found in the waste (concurrent enzymatic and microbial hydrolysis and fermentation) or found in recycled process wastes/solutions.

[0075] In some aspects the microbial growth has a pH lowering effect especially when metabolites like caboxylic acids and fatty acids (e.g. acetate, propionate, butyrate, lactate) is produced.

[0076] In other aspects of the disclosure it might be an advantage to inoculate MSW using different microbial species. These might include microorganisms that shows extra-cellular cellulase activities, microorganisms capable of degrading lignin, acetate-producing microorganisms, propionate-producing microorganisms, butyrate-producing organisms, ethanol-producing microorganisms and lactate producing microorganisms. Such embodiments are further described in DONG patent page 21-25 (WO2013/185777)

[0077] In practicing embodiments of the invention it can be advantageous to adjust temperature and water and dry matter content of the MSW. Enzymes normally show an optimal temperature and dry matter range. Hydrolysis of MSW is normally performed with agitation. This can be in reactors providing agitation by free fall mixing (as also described by DONG WO2006/056838 and WO2011/032557), stirred-tank reactors or similar systems. Suitable process time, temperature and pH conditions can readily be determined by one skilled in the art and is dependent on MSW composition, dry matter concentration and enzyme.

[0078] The present disclosure also relates to processes for degrading a municipal solid waste material, comprising: treating the municipal solid waste material with an enzyme composition comprising a cellulolytic background composition combined with one or more enzymes selected from (i) a protease; (ii) a lipase and (iii) a beta-glucanase; and optionally combined with one or more further enzymes selected from (iv) a pectate lyase; (v) a mannanase and (vi) an amylase. In one aspect, the processes further comprise recovering the degraded municipal solid waste material. Soluble products of degradation of the municipal solid waste material can be separated from insoluble municipal solid waste material using a method known in the art such as, for example, centrifugation, filtration, or gravity settling. In preferred aspects solubilized compounds can be converted to Biogas (mainly comprising CH₄ and CO₂) by anaerobic digestion. In other aspects solubilized sugars can be converted to ethanol by fermentation.

[0079] The present disclosure also relates to processes of producing a fermentation product, comprising: (a) solubilizing a municipal solid waste material with an enzyme composition comprising a cellulolytic background composition combined with one or more enzymes selected from (i) a protease; (ii) a lipase and (iii) a beta-glucanase; and optionally combined with one or more further enzymes selected from (iv) a pectate lyase; (v) a mannanase and (vi) an amylase; (b) fermenting the solubilized municipal solid waste material with one or more (e.g., several) fermenting microorganisms to produce the fermentation product; and (c) recovering the fermentation product from the fermentation. In preferred embodiments solubilized compounds can be converted to biogas (mainly comprising CH₄ and CO₂) by anaerobic digestion. In other aspects solubilized sugars can be converted to ethanol by fermentation.

[0080] The present disclosure also relates to processes of fermenting a municipal solid waste material, comprising: fermenting the municipal solid waste material with one or more (e.g., several) fermenting microorganisms, wherein the municipal solid waste material is saccharified with an enzyme composition comprising a cellulolytic background composition combined with one or more enzymes selected from (i) a protease; (ii) a lipase and (iii) a beta-glucanase; and optionally combined with one or more further enzymes selected from (iv) a pectate lyase; (v) a mannanase and (vi) an amylase. In one aspect, the fermenting of the municipal solid waste material produces a fermentation product. In another aspect, the processes further comprise recovering the fermentation product from the fermentation. In preferred aspects solubilized compounds can be converted to Biogas (mainly comprising CH₄ and CO₂) by anaerobic digestion. In other aspects solubilized sugars can be converted to ethanol by fermentation.

[0081] The processes of the present disclosure can also be used to solubilize the municipal solid waste material to fermentable sugars and to convert the fermentable sugars to many useful fermentation products, *e.g.*, fuel (ethanol, n-butanol, isobutanol, biodiesel, jet fuel) and/or platform chemicals (*e.g.*, acids, alcohols, ketones, gases, oils, and the like). The production of a desired fermentation product from the municipal solid waste material typically involves enzymatic solubilization and fermentation.

[0082] The processing of the municipal solid waste material according to the present disclosure can be accomplished using methods conventional in the art. Moreover, the processes of the present invention can be implemented using any conventional biomass processing apparatus configured to operate in accordance with the invention.

[0083] Solubilization and fermentation, separate or simultaneous, include, but are not limited to, separate hydrolysis and fermentation (SHF); simultaneous saccharification and fermentation (SSF); simultaneous saccharification and co-fermentation (SSCF); hybrid hydrolysis and fermentation (HHF); separate hydrolysis and co-fermentation (SHCF); hybrid hydrolysis and co-fermentation (HHCF); and direct microbial conversion (DMC), also sometimes called consolidated bioprocessing (CBP). SHF uses separate process steps to first enzymatically hydrolyze the municipal solid waste material to fermentable sugars, *e.g.*, glucose, cellobiose, and pentose monomers, and then ferment the fermentable sugars to ethanol. In SSF, the enzymatic hydrolysis of the municipal solid waste material and the fermentation of sugars to ethanol are combined in one step (Philippidis, G. P., 1996, Cellulose bioconversion technology, in Handbook on Bioethanol: Production and Utilization, Wyman, C. E., ed., Taylor & Francis, Washington, DC, 179-212). SSCF involves the co-fermentation of multiple sugars (Sheehan and Himmel, 1999, Biotechnol. Prog. 15: 817-827). HHF involves a separate hydrolysis step, and in addition a simultaneous saccharification and hydrolysis step, which can be carried out in the same reactor. The steps in an HHF process can be carried out at different temperatures, *i.e.*, high temperature enzymatic saccharification followed by SSF at a lower temperature that the fermentation strain can tolerate. DMC combines all three processes (enzyme production, hydrolysis, and fermentation) in one or more (*e.g.*, several) steps where the same organism is used to produce the enzymes for conversion of the municipal solid waste material to fermentable sugars and to convert the fermentable sugars into a final product (Lynd et al., 2002, Microbiol. Mol. Biol. Reviews 66: 506-577). It is understood herein that any method known in the art comprising pretreatment, enzymatic hydrolysis, fermentation, or a combination thereof, can be used in the practicing the processes of the present invention.

[0084] A conventional apparatus can include a fed-batch stirred reactor, a batch stirred reactor, a continuous flow stirred reactor with ultrafiltration, and/or a continuous plug-flow column reactor (de Castilhos Corazza et al., 2003, Acta Scientiarum. Technology 25: 33-38; Gusakov and Sinitsyn, 1985, Enz. Microb. Technol. 7: 346-352), an attrition reactor (Ryu and Lee, 1983, Biotechnol. Bioeng. 25: 53-65). Additional reactor types include fluidized bed, upflow blanket, immobilized, and extruder type reactors for hydrolysis and/or fermentation.

[0085] Pretreatment. In practicing the processes of the present invention, any pretreatment process known in the art can be used to disrupt plant cell wall components of the municipal solid waste material (Chandra et al., 2007, Adv. Biochem. Engin./Biotechnol. 108: 67-93; Galbe and Zacchi, 2007, Adv. Biochem. Engin./Biotechnol. 108: 41-65; Hendriks and Zeeman, 2009, Bioresource Technology 100: 10-18; Mosier et al., 2005, Bioresource Technology 96: 673-686; Taherzadeh and Karimi, 2008, Int. J. Mol. Sci. 9: 1621-1651; Yang and Wyman, 2008, Biofuels Bioproducts and Biorefining-Biofpr. 2: 26-40).

[0086] In a preferred aspects of the disclosure MSW is subject to a mild to severe temperature pretreatment in the range 10-300°C prior to hydrolysis. Heating will normally occur together with a mixing. Heating will normally be carried out by addition of water or steam. Pretreatment might also consist of a separation (manual or automatic) of MSW in different fractions. The municipal solid waste material can also be subjected to particle size reduction, sieving, pre-soaking, wetting, washing, and/or conditioning prior to pretreatment using methods known in the art.

[0087] Conventional pretreatments include, but are not limited to, steam pretreatment (with or without explosion), dilute acid pretreatment, hot water pretreatment, alkaline pretreatment, lime pretreatment, wet oxidation, wet explosion, ammonia fiber explosion, organosolv pretreatment, and biological pretreatment. Additional pretreatments include ammonia percolation, ultrasound, electroporation, microwave, supercritical CO₂, supercritical H₂O, ozone, ionic liquid, and gamma irradiation pretreatments.

[0088] The municipal solid waste material can be pretreated before hydrolysis and/or fermentation. Pretreatment is preferably performed prior to the hydrolysis. Alternatively, the pretreatment can be carried out simultaneously with enzyme hydrolysis to release fermentable sugars, such as glucose, xylose, and/or cellobiose. In most cases the pretreatment step itself results in some conversion of biomass to fermentable sugars (even in absence of enzymes).

[0089] Mechanical Pretreatment or Physical Pretreatment: The term "mechanical pretreatment" or "physical pretreatment" refers to any pretreatment that promotes size reduction of particles. For example, such pretreatment can involve various types of grinding or milling (*e.g.*, dry milling, wet milling, or vibratory ball milling).

[0090] The municipal solid waste material can be pretreated both physically (mechanically) and chemically. Mechanical or physical pretreatment can be coupled with steaming/steam explosion, hydrothermolysis, dilute or mild acid treatment, high temperature, high pressure treatment, irradiation (*e.g.*, microwave irradiation), or combinations thereof. In one aspect, high pressure means pressure in the range of preferably about 100 to about 400 psi, *e.g.*, about 150 to about

250 psi. In another aspect, high temperature means temperature in the range of about 100 to about 300°C, e.g., about 140 to about 200°C. In a preferred aspect, mechanical or physical pretreatment is performed in a batch-process using a steam gun hydrolyzer system that uses high pressure and high temperature as defined above, e.g., a Sunds Hydrolyzer available from Sunds Defibrator AB, Sweden. The physical and chemical pretreatments can be carried out sequentially or simultaneously, as desired.

[0091] Accordingly, in a preferred aspect, the municipal solid waste material is subjected to physical (mechanical) or chemical pretreatment, or any combination thereof, to promote the separation and/or release of cellulose, hemicellulose, and/or lignin.

[0092] Biological Pretreatment. The term "biological pretreatment" refers to any biological pretreatment that promotes the separation and/or release of cellulose, hemicellulose, and/or lignin from the municipal solid waste material. Biological pretreatment techniques can involve applying lignin-solubilizing microorganisms and/or enzymes (see, for example, Hsu, T.-A., 1996, Pretreatment of biomass, in Handbook on Bioethanol: Production and Utilization, Wyman, C. E., ed., Taylor & Francis, Washington, DC, 179-212; Ghosh and Singh, 1993, Adv. Appl. Microbiol. 39: 295-333; McMillan, J. D., 1994, Pretreating lignocellulosic biomass: a review, in Enzymatic Conversion of Biomass for Fuels Production, Himmel, M. E., Baker, J. O., and Overend, R. P., eds., ACS Symposium Series 566, American Chemical Society, Washington, DC, chapter 15; Gong, C. S., Cao, N. J., Du, J., and Tsao, G. T., 1999, Ethanol production from renewable resources, in Advances in Biochemical Engineering/Biotechnology, Scheper, T., ed., Springer-Verlag Berlin Heidelberg, Germany, 65: 207-241; Olsson and Hahn-Hagerdal, 1996, Enz. Microb. Tech. 18: 312-331; and Vallander and Eriksson, 1990, Adv. Biochem. Eng./Biotechnol. 42: 63-95).

[0093] In other aspects MSW can be pretreated both physically (mechanically) and chemically. Mechanical or physical pretreatment can be coupled with steaming/steam explosion, hydrothermolysis, dilute or mild acid treatment, high temperature, high pressure treatment, irradiation (e.g., microwave irradiation), or combinations thereof. In one aspect, high pressure means pressure in the range of preferably about 100 to about 400 psi, e.g., about 150 to about 250 psi. In another aspect, high temperature means temperature in the range of about 100 to about 300°C, e.g., about 140 to about 200°C. In a preferred aspect, mechanical or physical pretreatment is performed in a batch-process using a steam gun hydrolyzer system that uses high pressure and high temperature as defined above, e.g., a Sunds Hydrolyzer available from Sunds Defibrator AB, Sweden. The physical and chemical pretreatments can be carried out sequentially or simultaneously, as desired.

[0094] Hydrolysis. In the hydrolysis step, the municipal solid waste material, e.g., pretreated, is hydrolyzed to break down cellulose and/or hemicellulose and other substrates to fermentable sugars, such as glucose, cellobiose, xylose, xylulose, arabinose, mannose, galactose, and/or soluble oligosaccharides (also known as saccharification). The hydrolysis is performed enzymatically by one or more enzyme compositions in one or more stages. In the hydrolysis step, the municipal solid waste material, e.g., pretreated, is hydrolyzed to break down proteins and lipids (e.g. triglycerides) found in the waste.

[0095] The hydrolysis can be carried out as a batch process or series of batch processes. The hydrolysis can be carried out as a fed batch or continuous process, or series of fed batch or continuous processes, where the municipal solid waste material is fed gradually to, for example, a hydrolysis solution containing an enzyme composition. In an embodiment the hydrolysis a continuous hydrolysis in which a MSW material and a enzymes composition are added at different intervals throughout the hydrolysis and the hydrolysate is removed at different intervals throughout the hydrolysis. The removal of the hydrolysate may occur prior to, simultaneously with, or after the addition of the cellulosic material and the cellulolytic enzymes composition.

[0096] Enzymatic hydrolysis is preferably carried out in a suitable aqueous environment under conditions that can be readily determined by one skilled in the art. In one aspect, hydrolysis is performed under conditions suitable for the activity of the enzymes(s), i.e., optimal for the enzyme(s).

[0097] In one aspect, the saccharification is performed in the presence of dissolved oxygen at a concentration of at least 0.5% of the saturation level.

[0098] In an aspect of the disclosure the dissolved oxygen concentration during saccharification is in the range of at least 0.5% up to 30% of the saturation level, such as at least 1% up to 25%, at least 1% up to 20%, at least 1% up to 15%, at least 1% up to 10%, at least 1% up to 5%, and at least 1% up to 3%. In a preferred embodiment, the dissolved oxygen concentration is maintained at a concentration of at least 0.5% up to 30% of the saturation level, such as at least 1% up to 25%, at least 1% up to 20%, at least 1% up to 15%, at least 1% up to 10%, at least 1% up to 5%, and at least 1% up to 3% during at least 25%, such as at least 50% or at least 75% of the saccharification period. When the enzyme composition comprises an oxidoreductase the dissolved oxygen concentration may be higher up to 70% of the saturation level.

[0099] Oxygen is added to the vessel in order to achieve the desired concentration of dissolved oxygen during saccharification. Maintaining the dissolved oxygen level within a desired range can be accomplished by aeration of the vessel, tank or the like by adding compressed air through a diffuser or sparger, or by other known methods of aeration. The aeration rate can be controlled on the basis of feedback from a dissolved oxygen sensor placed in the vessel/tank,

or the system can run at a constant rate without feedback control. In the case of a hydrolysis train consisting of a plurality of vessels/tanks connected in series, aeration can be implemented in one or more or all of the vessels/tanks. Oxygen aeration systems are well known in the art. According to the invention any suitable aeration system may be used. Commercial aeration systems are designed by, e.g., Chemineer, Derby, England, and build by, e.g., Paul Mueller Company, MO, USA.

[0100] The enzyme compositions can comprise any protein useful in degrading the municipal solid waste material.

[0101] In one aspect, the enzyme composition comprises or further comprises one or more (e.g., several) proteins selected from the group consisting of a cellulase, an AA9 polypeptide, a hemicellulase, an esterase, an expansin, a ligninolytic enzyme, an oxidoreductase, a pectinase, a protease, and a swollenin. In another aspect, the cellulase is preferably one or more (e.g., several) enzymes selected from the group consisting of an endoglucanase, a cellobiohydrolase, and a beta-glucosidase. In another aspect, the hemicellulase is preferably one or more (e.g., several) enzymes selected from the group consisting of an acetylmannan esterase, an acetylxylan esterase, an arabinanase, an arabinofuranosidase, a coumaric acid esterase, a feruloyl esterase, a galactosidase, a glucuronidase, a glucuronoyl esterase, a mannanase, a mannosidase, a xylanase, and a xylosidase. In another aspect, the oxidoreductase is preferably one or more (e.g., several) enzymes selected from the group consisting of a catalase, a laccase, and a peroxidase.

[0102] In another aspect, the enzyme composition comprises one or more (e.g., several) cellulolytic enzymes. In another aspect, the enzyme composition comprises or further comprises one or more (e.g., several) hemicellulolytic enzymes. In another aspect, the enzyme composition comprises one or more (e.g., several) cellulolytic enzymes and one or more (e.g., several) hemicellulolytic enzymes. In another aspect, the enzyme composition comprises one or more (e.g., several) enzymes selected from the group of cellulolytic enzymes and hemicellulolytic enzymes. In another aspect, the enzyme composition comprises an endoglucanase. In another aspect, the enzyme composition comprises a cellobiohydrolase. In another aspect, the enzyme composition comprises a beta-glucosidase. In another aspect, the enzyme composition comprises an AA9 polypeptide. In another aspect, the enzyme composition comprises an endoglucanase and an AA9 polypeptide. In another aspect, the enzyme composition comprises a cellobiohydrolase and an AA9 polypeptide. In another aspect, the enzyme composition comprises a beta-glucosidase and an AA9 polypeptide. In another aspect, the enzyme composition comprises an endoglucanase and a cellobiohydrolase. In another aspect, the enzyme composition comprises an endoglucanase and a cellobiohydrolase I, a cellobiohydrolase II, or a combination of a cellobiohydrolase I and a cellobiohydrolase II. In another aspect, the enzyme composition comprises an endoglucanase and a beta-glucosidase. In another aspect, the enzyme composition comprises a beta-glucosidase and a cellobiohydrolase. In another aspect, the enzyme composition comprises a beta-glucosidase and a cellobiohydrolase I, a cellobiohydrolase II, or a combination of a cellobiohydrolase I and a cellobiohydrolase II. In another aspect, the enzyme composition comprises an endoglucanase, an AA9 polypeptide, and a cellobiohydrolase. In another aspect, the enzyme composition comprises an endoglucanase, an AA9 polypeptide, and a cellobiohydrolase I, a cellobiohydrolase II, or a combination of a cellobiohydrolase I and a cellobiohydrolase II. In another aspect, the enzyme composition comprises an endoglucanase, a beta-glucosidase, and an AA9 polypeptide. In another aspect, the enzyme composition comprises a beta-glucosidase, an AA9 polypeptide, and a cellobiohydrolase. In another aspect, the enzyme composition comprises a beta-glucosidase, an AA9 polypeptide, and a cellobiohydrolase I, a cellobiohydrolase II, or a combination of a cellobiohydrolase I and a cellobiohydrolase II. In another aspect, the enzyme composition comprises an endoglucanase, a beta-glucosidase, and a cellobiohydrolase. In another aspect, the enzyme composition comprises an endoglucanase, a beta-glucosidase, and a cellobiohydrolase I, a cellobiohydrolase II, or a combination of a cellobiohydrolase I and a cellobiohydrolase II. In another aspect, the enzyme composition comprises an endoglucanase, a beta-glucosidase, and an AA9 polypeptide. In another aspect, the enzyme composition comprises an endoglucanase, a beta-glucosidase, an AA9 polypeptide, and a cellobiohydrolase I, a cellobiohydrolase II, or a combination of a cellobiohydrolase I and a cellobiohydrolase II.

[0103] In another aspect, the enzyme composition comprises an acetylmannan esterase. In another aspect, the enzyme composition comprises an acetylxylan esterase. In another aspect, the enzyme composition comprises an arabinanase (e.g., alpha-L-arabinanase). In another aspect, the enzyme composition comprises an arabinofuranosidase (e.g., alpha-L-arabinofuranosidase). In another aspect, the enzyme composition comprises a coumaric acid esterase. In another aspect, the enzyme composition comprises a feruloyl esterase. In another aspect, the enzyme composition comprises a galactosidase (e.g., alpha-galactosidase and/or beta-galactosidase). In another aspect, the enzyme composition comprises a glucuronidase (e.g., alpha-D-glucuronidase). In another aspect, the enzyme composition comprises a glucuronoyl esterase. In another aspect, the enzyme composition comprises a mannanase. In another aspect, the enzyme composition comprises a mannosidase (e.g., beta-mannosidase). In another aspect, the enzyme composition comprises a xylanase. In an embodiment, the xylanase is a Family 10 xylanase. In another embodiment, the xylanase is a Family 11 xylanase. In another aspect, the enzyme composition comprises a xylosidase (e.g., beta-xylosidase).

[0104] In another aspect, the enzyme composition comprises an esterase. In another aspect, the enzyme composition comprises an expansin. In another aspect, the enzyme composition comprises a ligninolytic enzyme. In an embodiment, the ligninolytic enzyme is a manganese peroxidase. In another embodiment, the ligninolytic enzyme is a lignin peroxidase.

In another embodiment, the ligninolytic enzyme is a H₂O₂-producing enzyme. In another aspect, the enzyme composition comprises a pectinase. In another aspect, the enzyme composition comprises an oxidoreductase. In an embodiment, the oxidoreductase is a catalase. In another embodiment, the oxidoreductase is a laccase. In another embodiment, the oxidoreductase is a peroxidase. In another aspect, the enzyme composition comprises a protease. In another aspect,

[0105] In the processes of the present invention, the enzyme(s) can be added prior to or during saccharification, saccharification and fermentation, or fermentation.

[0106] One or more (e.g., several) components of the enzyme composition may be native proteins, recombinant proteins, or a combination of native proteins and recombinant proteins. For example, one or more (e.g., several) components may be native proteins of a cell, which is used as a host cell to express recombinantly one or more (e.g., several) other components of the enzyme composition. It is understood herein that the recombinant proteins may be heterologous (e.g., foreign) and/or native to the host cell. One or more (e.g., several) components of the enzyme composition may be produced as monocomponents, which are then combined to form the enzyme composition. The enzyme composition may be a combination of multicomponent and monocomponent protein preparations.

[0107] The enzymes used in the processes of the present invention may be in any form suitable for use, such as, for example, a fermentation broth formulation or a cell composition, a cell lysate with or without cellular debris, a semi-purified or purified enzyme preparation, or a host cell as a source of the enzymes. The enzyme composition may be a dry powder or granulate, a non-dusting granulate, a liquid, a stabilized liquid, or a stabilized protected enzyme. Liquid enzyme preparations may, for instance, be stabilized by adding stabilizers such as a sugar, a sugar alcohol or another polyol, and/or lactic acid or another organic acid according to established processes.

[0108] The optimum amounts of the enzymes and polypeptides having enzymatic activity depend on several factors including, but not limited to, the mixture of cellulolytic enzymes and/or hemicellulolytic enzymes, the municipal solid waste material, the concentration of municipal solid waste material, the pretreatment(s) of the municipal solid waste material, temperature, time, pH, and inclusion of a fermenting organism (e.g., for Simultaneous Saccharification and Fermentation).

[0109] In one aspect, an effective amount of the enzyme composition to the municipal solid waste material is about 0.5 to about 50 mg, e.g., about 0.5 to about 40 mg, about 0.5 to about 25 mg, about 0.75 to about 20 mg, about 0.75 to about 15 mg, about 0.5 to about 10 mg, or about 2.5 to about 10 mg per g of the municipal solid waste material. In a related aspect, the protease is present at a ratio between 0-20% w/w, such as e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 % w/w of the total enzyme protein.

[0110] In one aspect, the beta-glucanase is present at a ratio between 0-30% w/w, such as e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30% w/w of the total enzyme protein. In a related aspect, the pectate-lyase is present at a ratio between 0-10% w/w, such as e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10% w/w of the total enzyme protein. In a related aspect, the mannanase or amylase is present at a ratio between 0-10% w/w, such as e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10% w/w of the total enzyme protein. In yet another related aspect, the cellulolytic enzyme blend is present at a ratio between 40%-99% w/w, such as e.g. between 50%-90% w/w, such as e.g. 60%-80% w/w, such as e.g. 65-75% of the total enzyme protein.

[0111] The polypeptides having cellulolytic enzyme activity or hemicellulolytic enzyme activity as well as other proteins/polypeptides useful in the degradation of the municipal solid waste material, e.g., AA9 polypeptides can be derived or obtained from any suitable origin, including, archaeal, bacterial, fungal, yeast, plant, or animal origin. The term "obtained" also means herein that the enzyme may have been produced recombinantly in a host organism employing methods described herein, wherein the recombinantly produced enzyme is either native or foreign to the host organism or has a modified amino acid sequence, e.g., having one or more (e.g., several) amino acids that are deleted, inserted and/or substituted, i.e., a recombinantly produced enzyme that is a mutant and/or a fragment of a native amino acid sequence or an enzyme produced by nucleic acid shuffling processes known in the art. Encompassed within the meaning of a native enzyme are natural variants and within the meaning of a foreign enzyme are variants obtained by, e.g., site-directed mutagenesis or shuffling.

[0112] Each polypeptide may be a bacterial polypeptide. For example, each polypeptide may be a Gram-positive bacterial polypeptide having enzyme activity, or a Gram-negative bacterial polypeptide having enzyme activity.

[0113] Each polypeptide may also be a fungal polypeptide, e.g., a yeast polypeptide or a filamentous fungal polypeptide.

[0114] Chemically modified or protein engineered mutants of polypeptides may also be used.

[0115] One or more (e.g., several) components of the enzyme composition may be a recombinant component, i.e., produced by cloning of a DNA sequence encoding the single component and subsequent cell transformed with the DNA sequence and expressed in a host (see, for example, WO 91/17243 and WO 91/17244). The host can be a heterologous host (enzyme is foreign to host), but the host may under certain conditions also be a homologous host (enzyme is native to host). Monocomponent cellulolytic proteins may also be prepared by purifying such a protein from a fermentation broth.

[0116] In one aspect, the one or more (e.g., several) cellulolytic enzymes comprise a commercial cellulolytic enzyme preparation. Examples of commercial cellulolytic enzyme preparations suitable for use in the present invention include,

for example, CELLIC® CTec (Novozymes A/S), CELLIC® CTec2 (Novozymes A/S), CELLIC® CTec3 (Novozymes A/S), CELLULAST® (Novozymes A/S), NOVOZYM™ 188 (Novozymes A/S), SPEZYME™ CP (Genencor Int.), ACCELERASE™ TRIO (DuPont), FILTRASE® NL (DSM); METHAPLUS® S/L 100 (DSM), ROHAMENT™ 7069 W (Röhm GmbH), or ALTERNAFUEL® CMAX3™ (Dyadic International, Inc.). The cellulolytic enzyme preparation is added in an amount effective from about 0.001 to about 5.0 wt. % of solids, e.g., about 0.025 to about 4.0 wt. % of solids or about 0.005 to about 2.0 wt. % of solids.

[0117] Examples of bacterial endoglucanases that can be used in the processes of the present invention, include, but are not limited to, *Acidothermus cellulolyticus* endoglucanase (WO 91/05039; WO 93/15186; U.S. Patent No. 5,275,944; WO 96/02551; U.S. Patent No. 5,536,655; WO 00/70031; WO 05/093050), *Erwinia carotovora* endoglucanase (Saarilahti et al., 1990, Gene 90: 9-14), *Thermobifida fusca* endoglucanase III (WO 05/093050), and *Thermobifida fusca* endoglucanase V (WO 05/093050).

[0118] Examples of fungal endoglucanases that can be used in the present invention, include, but are not limited to, *Trichoderma reesei* endoglucanase I (Penttilä et al., 1986, Gene 45: 253-263, *Trichoderma reesei* Cel7B endoglucanase I (GenBank:M15665), *Trichoderma reesei* endoglucanase II (Saloheimo et al., 1988, Gene 63:11-22), *Trichoderma reesei* Cel5A endoglucanase II (GenBank:M19373), *Trichoderma reesei* endoglucanase III (Okada et al., 1988, Appl. Environ. Microbiol. 64: 555-563, GenBank:AB003694), *Trichoderma reesei* endoglucanase V (Saloheimo et al., 1994, Molecular Microbiology 13: 219-228, GenBank:Z33381), *Aspergillus aculeatus* endoglucanase (Ooi et al., 1990, Nucleic Acids Research 18: 5884), *Aspergillus kawachii* endoglucanase (Sakamoto et al., 1995, Current Genetics 27: 435-439), *Fusarium oxysporum* endoglucanase (GenBank:L29381), *Humicola grisea* var. *thermoidea* endoglucanase (GenBank:AB003107), *Melanocarpus albomyces* endoglucanase (GenBank:MAL515703), *Neurospora crassa* endoglucanase (GenBank:XM_324477), *Humicola insolens* endoglucanase V, *Myceliophthora thermophila* CBS 117.65 endoglucanase, *Thermoascus aurantiacus* endoglucanase I (GenBank:AF487830), *Trichoderma reesei* strain No. VTT-D-80133 endoglucanase (GenBank:M15665), and *Penicillium pinophilum* endoglucanase (WO 2012/062220).

[0119] Examples of cellobiohydrolases useful in the present invention include, but are not limited to, *Aspergillus aculeatus* cellobiohydrolase II (WO 2011/059740), *Aspergillus fumigatus* cellobiohydrolase I (WO 2013/028928), *Aspergillus fumigatus* cellobiohydrolase II (WO 2013/028928), *Chaetomium thermophilum* cellobiohydrolase I, *Chaetomium thermophilum* cellobiohydrolase II, *Humicola insolens* cellobiohydrolase I, *Myceliophthora thermophila* cellobiohydrolase II (WO 2009/042871), *Penicillium occitanis* cellobiohydrolase I (GenBank:AY690482), *Talaromyces emersonii* cellobiohydrolase I (GenBank:AF439936), *Thielavia hyrcanie* cellobiohydrolase II (WO 2010/141325), *Thielavia terrestris* cellobiohydrolase II (CEL6A, WO 2006/074435), *Trichoderma reesei* cellobiohydrolase I, *Trichoderma reesei* cellobiohydrolase II, and *Trichophaea saccata* cellobiohydrolase II (WO 2010/057086).

[0120] Examples of beta-glucosidases useful in the present invention include, but are not limited to, beta-glucosidases from *Aspergillus aculeatus* (Kawaguchi et al., 1996, Gene 173: 287-288), *Aspergillus fumigatus* (WO 2005/047499), *Aspergillus niger* (Dan et al., 2000, J. Biol. Chem. 275: 4973-4980), *Aspergillus oryzae* (WO 02/095014), *Penicillium brasilianum* IBT 20888 (WO 2007/019442 and WO 2010/088387), *Thielavia terrestris* (WO 2011/035029), and *Trichophaea saccata* (WO 2007/019442).

[0121] Other useful endoglucanases, cellobiohydrolases, and beta-glucosidases are disclosed in numerous Glycosyl Hydrolase families using the classification according to Henrissat, 1991, Biochem. J. 280: 309-316, and Henrissat and Bairoch, 1996, Biochem. J. 316: 695-696.

[0122] In the processes of the present invention, any AA9 polypeptide can be used as a component of the enzyme composition.

[0123] Examples of AA9 polypeptides useful in the processes of the present invention include, but are not limited to, AA9 polypeptides from *Thielavia terrestris* (WO 2005/074647, WO 2008/148131, and WO 2011/035027), *Thermoascus aurantiacus* (WO 2005/074656 and WO 2010/065830), *Trichoderma reesei* (WO 2007/089290 and WO 2012/149344), *Myceliophthora thermophila* (WO 2009/085935, WO 2009/085859, WO 2009/085864, WO 2009/085868, and WO 2009/033071), *Aspergillus fumigatus* (WO 2010/138754), *Penicillium pinophilum* (WO 2011/005867), *Thermoascus* sp. (WO 2011/039319), *Penicillium* sp. (*emersonii*) (WO 2011/041397 and WO 2012/000892), *Thermoascus crustaceus* (WO 2011/041504), *Aspergillus aculeatus* (WO 2012/125925), *Thermomyces lanuginosus* (WO 2012/113340, WO 2012/129699, WO 2012/130964, and WO 2012/129699), *Aurantiporus alborubescens* (WO 2012/122477), *Trichophaea saccata* (WO 2012/122477), *Penicillium thomii* (WO 2012/122477), *Talaromyces stipitatus* (WO 2012/135659), *Humicola insolens* (WO 2012/146171), *Malbranchea cinnamomea* (WO 2012/101206), *Talaromyces leycettanus* (WO 2012/101206), and *Chaetomium thermophilum* (WO 2012/101206), and *Talaromyces thermophilus* (WO 2012/129697 and WO 2012/130950).

[0124] In one aspect, the AA9 polypeptide is used in the presence of a soluble activating divalent metal cation according to WO 2008/151043, e.g., manganese or copper.

[0125] In another aspect, the AA9 polypeptide is used in the presence of a dioxy compound, a bicyclic compound, a heterocyclic compound, a nitrogen-containing compound, a quinone compound, a sulfur-containing compound, or a liquor obtained from a pretreated municipal solid waste material such as pretreated corn stover (WO 2012/021394, WO

2012/021395, WO 2012/021396, WO 2012/021399, WO 2012/021400, WO 2012/021401, WO 2012/021408, and WO 2012/021410).

[0126] The term "liquor" means the solution phase, either aqueous, organic, or a combination thereof, arising from treatment of a lignocellulose and/or hemicellulose material in a slurry, or monosaccharides thereof, e.g., xylose, arabinose, mannose, etc., under conditions as described in WO 2012/021401, and the soluble contents thereof. A liquor for cellulolytic enhancement of an AA9 polypeptide can be produced by treating a lignocellulose or hemicellulose material (or feedstock) by applying heat and/or pressure, optionally in the presence of a catalyst, e.g., acid, optionally in the presence of an organic solvent, and optionally in combination with physical disruption of the material, and then separating the solution from the residual solids. Such conditions determine the degree of cellulolytic enhancement obtainable through the combination of liquor and an AA9 polypeptide during hydrolysis of a cellulosic substrate by a cellulolytic enzyme preparation. The liquor can be separated from the treated material using a method standard in the art, such as filtration, sedimentation, or centrifugation.

[0127] In one aspect, an effective amount of the liquor to cellulose is about 10^{-6} to about 10 g per g of cellulose, e.g., about 10^{-6} to about 7.5 g, about 10^{-6} to about 5 g, about 10^{-6} to about 2.5 g, about 10^{-6} to about 1 g, about 10^{-5} to about 1 g, about 10^{-5} to about 10^{-1} g, about 10^{-4} to about 10^{-1} g, about 10^{-3} to about 10^{-1} g, or about 10^{-3} to about 10^{-2} g per g of cellulose.

[0128] In one aspect, the one or more (e.g., several) hemicellulolytic enzymes comprise a commercial hemicellulolytic enzyme preparation. Examples of commercial hemicellulolytic enzyme preparations suitable for use in the present invention include, for example, SHEARZYME™ (Novozymes A/S), CELLIC® HTec (Novozymes A/S), CELLIC® HTec2 (Novozymes A/S), CELLIC® HTec3 (Novozymes A/S), VISCOZYME® (Novozymes A/S), ULTRAFLO® (Novozymes A/S), PULPZYME® HC (Novozymes A/S), MULTIFECT® Xylanase (Genencor), ACCELLERASE® XY (Genencor), ACCELLERASE® XC (Genencor), ECOPULP® TX-200A (AB Enzymes), HSP 6000 Xylanase (DSM), DEPOL™ 333P (Biocatalysts Limit, Wales, UK), DEPOL™ 740L (Biocatalysts Limit, Wales, UK), and DEPOL™ 762P (Biocatalysts Limit, Wales, UK), ALTERNA FUEL 100P (Dyadic), and ALTERNA FUEL 200P (Dyadic).

[0129] Examples of xylanases useful in the processes of the present invention include, but are not limited to, xylanases from *Aspergillus aculeatus* (GeneSeqP:AAR63790; WO 94/21785), *Aspergillus fumigatus* (WO 2006/078256), *Penicillium pinophilum* (WO 2011/041405), *Penicillium* sp. (WO 2010/126772), *Thermomyces lanuginosus* (GeneSeqP:BAA22485), *Talaromyces thermophilus* (GeneSeqP:BAA22834), *Thielavia terrestris* NRRL 8126 (WO 2009/079210), and *Trichophaea saccata* (WO 2011/057083).

[0130] Examples of beta-xylosidases useful in the processes of the present invention include, but are not limited to, beta-xylosidases from *Neurospora crassa* (SwissProt:Q7SOW4), *Trichoderma reesei* (UniProtKB/TrEMBL:Q92458), *Talaromyces emersonii* (SwissProt:Q8X212), and *Talaromyces thermophilus* (GeneSeqP:BAA22816).

[0131] Examples of acetylxyylan esterases useful in the processes of the present invention include, but are not limited to, acetylxyylan esterases from *Aspergillus aculeatus* (WO 2010/108918), *Chaetomium globosum* (UniProt:Q2GWX4), *Chaetomium gracile* (GeneSeqP:AAB82124), *Humicola insolens* DSM 1800 (WO 2009/073709), *Hypocrea jecorina* (WO 2005/001036), *Myceliophthora thermophila* (WO 2010/014880), *Neurospora crassa* (UniProt:q7s259), *Phaeosphaeria nodorum* (UniProt:Q0UJH1), and *Thielavia terrestris* NRRL 8126 (WO 2009/042846).

[0132] Examples of feruloyl esterases (ferulic acid esterases) useful in the processes of the present invention include, but are not limited to, feruloyl esterases from *Humicola insolens* DSM 1800 (WO 2009/076122), *Neosartorya fischeri* (UniProt:A1D9T4), *Neurospora crassa* (UniProt:Q9HGR3), *Penicillium aurantiogriseum* (WO 2009/127729), and *Thielavia terrestris* (WO 2010/053838 and WO 2010/065448).

[0133] Examples of arabinofuranosidases useful in the processes of the present invention include, but are not limited to, arabinofuranosidases from *Aspergillus niger* (GeneSeqP:AAR94170), *Humicola insolens* DSM 1800 (WO 2006/114094 and WO 2009/073383), and *M. giganteus* (WO 2006/114094).

[0134] Examples of alpha-glucuronidases useful in the processes of the present invention include, but are not limited to, alpha-glucuronidases from *Aspergillus clavatus* (UniProt:alcc12), *Aspergillus fumigatus* (SwissProt:Q4WW45), *Aspergillus niger* (UniProt:Q96WX9), *Aspergillus terreus* (SwissProt:Q0CJP9), *Humicola insolens* (WO 2010/014706), *Penicillium aurantiogriseum* (WO 2009/068565), *Talaromyces emersonii* (UniProt:Q8X211), and *Trichoderma reesei* (UniProt:Q99024).

[0135] Examples of oxidoreductases useful in the processes of the present invention include, but are not limited to, *Aspergillus lentilus* catalase, *Aspergillus fumigatus* catalase, *Aspergillus niger* catalase, *Aspergillus oryzae* catalase, *Humicola insolens* catalase, *Neurospora crassa* catalase, *Penicillium emersonii* catalase, *Scytalidium thermophilum* catalase, *Talaromyces stipitatus* catalase, *Thermoascus aurantiacus* catalase, *Coprinus cinereus* laccase, *Myceliophthora thermophila* laccase, *Polyporus pinsitus* laccase, *Pycnoporus cinnabarinus* laccase, *Rhizoctonia solani* laccase, *Streptomyces coelicolor* laccase, *Coprinus cinereus* peroxidase, Soy peroxidase, Royal palm peroxidase.

[0136] The polypeptides having enzyme activity used in the processes of the present invention may be produced by fermentation of the above-noted microbial strains on a nutrient medium containing suitable carbon and nitrogen sources and inorganic salts, using procedures known in the art (see, e.g., Bennett, J.W. and LaSure, L. (eds.), More Gene

Manipulations in Fungi, Academic Press, CA, 1991). Suitable media are available from commercial suppliers or may be prepared according to published compositions (e.g., in catalogues of the American Type Culture Collection). Temperature ranges and other conditions suitable for growth and enzyme production are known in the art (see, e.g., Bailey, J.E., and Ollis, D.F., Biochemical Engineering Fundamentals, McGraw-Hill Book Company, NY, 1986).

[0137] The fermentation can be any method of cultivation of a cell resulting in the expression or isolation of an enzyme or protein. Fermentation may, therefore, be understood as comprising shake flask cultivation, or small- or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermentors performed in a suitable medium and under conditions allowing the enzyme to be expressed or isolated. The resulting enzymes produced by the methods described above may be recovered from the fermentation medium and purified by conventional procedures.

[0138] Fermentation. In preferred aspects, some fermentation will occur concurrent with the hydrolysis of the MSW. Fermentable sugars obtained from the hydrolyzed municipal solid waste material can be fermented by one or more (e.g., several) fermenting microorganisms capable of fermenting the sugars directly or indirectly into fermentation products such as volatile fatty acids (e.g. acetate, propionate, butyrate), lactate and alcohols.

[0139] "Fermentation" or "fermentation process" refers to any fermentation process or any process comprising a fermentation step. Fermentation processes also include fermentation processes used in the consumable alcohol industry (e.g., beer and wine), dairy industry (e.g., fermented dairy products), leather industry, and tobacco industry. The fermentation conditions depend on the desired fermentation product and fermenting organism and can easily be determined by one skilled in the art.

[0140] In the fermentation step, sugars, released from the municipal solid waste material as a result of the pretreatment and enzymatic hydrolysis steps, are fermented to a product, e.g., ethanol, by a fermenting organism, such as yeast. Hydrolysis and fermentation can be separate or simultaneous.

[0141] Any suitable hydrolyzed municipal solid waste material can be used in the fermentation step in practicing the present invention. The material is generally selected based on economics, i.e., costs per equivalent sugar potential, and recalcitrance to enzymatic conversion.

[0142] The term "fermentation medium" is understood herein to refer to a medium before the fermenting microorganism(s) is(are) added, such as, a medium resulting from a saccharification process, as well as a medium used in a simultaneous saccharification and fermentation process (SSF).

[0143] "Fermenting microorganism" refers to any microorganism, including bacterial and fungal organisms, suitable for use in a desired fermentation process to produce a fermentation product. The fermenting organism can be hexose and/or pentose fermenting organisms, or a combination thereof. Both hexose and pentose fermenting organisms are well known in the art. Suitable fermenting microorganisms are able to ferment, i.e., convert, sugars, such as glucose, xylose, xylulose, arabinose, maltose, mannose, galactose, and/or oligosaccharides, directly or indirectly into the desired fermentation product. Examples of bacterial and fungal fermenting organisms producing ethanol are described by Lin et al., 2006, Appl. Microbiol. Biotechnol. 69: 627-642.

[0144] Examples of fermenting microorganisms that can ferment hexose sugars include bacterial and fungal organisms, such as yeast. Yeast include strains of *Candida*, *Kluyveromyces*, and *Saccharomyces*, e.g., *Candida sonorensis*, *Kluyveromyces marxianus*, and *Saccharomyces cerevisiae*.

[0145] Examples of fermenting organisms that can ferment pentose sugars in their native state include bacterial and fungal organisms, such as some yeast. Xylose fermenting yeast include strains of *Candida*, preferably *C. sheatae* or *C. sonorensis*; and strains of *Pichia*, e.g., *P. stipitis*, such as *P. stipitis* CBS 5773. Pentose fermenting yeast include strains of *Pachysolen*, preferably *P. tannophilus*. Organisms not capable of fermenting pentose sugars, such as xylose and arabinose, may be genetically modified to do so by methods known in the art.

[0146] Examples of bacteria that can efficiently ferment hexose and pentose to ethanol include, for example, *Bacillus coagulans*, *Clostridium acetobutylicum*, *Clostridium thermocellum*, *Clostridium phytofermentans*, *Geobacillus* sp., *Thermoanaerobacter saccharolyticum*, and *Zymomonas mobilis* (Philippidis, G. P., 1996, Cellulose bioconversion technology, in Handbook on Bioethanol: Production and Utilization, Wyman, C. E., ed., Taylor & Francis, Washington, DC, 179-212).

[0147] Other fermenting organisms include strains of *Bacillus*, such as *Bacillus coagulans*; *Candida*, such as *C. sonorensis*, *C. methanosorbosa*, *C. diddensiae*, *C. parapsilosis*, *C. naedodendra*, *C. blankii*, *C. entomophila*, *C. brassicae*, *C. pseudotropicalis*, *C. boidinii*, *C. utilis*, and *C. sheatae*; *Clostridium*, such as *C. acetobutylicum*, *C. thermocellum*, and *C. phytofermentans*; *E. coli*, especially *E. coli* strains that have been genetically modified to improve the yield of ethanol; *Geobacillus* sp.; *Hansenula*, such as *Hansenula anomala*; *Klebsiella*, such as *K. oxytoca*; *Kluyveromyces*, such as *K. marxianus*, *K. lactis*, *K. thermotolerans*, and *K. fragilis*; *Schizosaccharomyces*, such as *S. pombe*; *Thermoanaerobacter*, such as *Thermoanaerobacter saccharolyticum*; and *Zymomonas*, such as *Zymomonas mobilis*.

[0148] In an aspect, the fermenting microorganism has been genetically modified to provide the ability to ferment pentose sugars, such as xylose utilizing, arabinose utilizing, and xylose and arabinose co-utilizing microorganisms.

[0149] In another aspect, the fermenting organism comprises one or more polynucleotides encoding one or more cellulolytic enzymes, hemicellulolytic enzymes, and accessory enzymes described herein.

[0150] It is well known in the art that the organisms described above can also be used to produce other substances, as described herein.

[0151] The fermenting microorganism is typically added to the degraded municipal solid waste material or hydrolysate and the fermentation is performed for about 8 to about 96 hours, e.g., about 24 to about 60 hours. The temperature is typically between about 26°C to about 60°C, e.g., about 32°C or 50°C, and about pH 3 to about pH 8, e.g., pH 4-5, 6, or 7.

[0152] In one aspect, the yeast and/or another microorganism are applied to the degraded municipal solid waste material and the fermentation is performed for about 12 to about 96 hours, such as typically 24-60 hours. In another aspect, the temperature is preferably between about 20°C to about 60°C, e.g., about 25°C to about 50°C, about 32°C to about 50°C, or about 32°C to about 50°C, and the pH is generally from about pH 3 to about pH 7, e.g., about pH 4 to about pH 7. However, some fermenting organisms, e.g., bacteria, have higher fermentation temperature optima. Yeast or another microorganism is preferably applied in amounts of approximately 10^5 to 10^{12} , preferably from approximately 10^7 to 10^{10} , especially approximately 2×10^8 viable cell count per ml of fermentation broth. Further guidance in respect of using yeast for fermentation can be found in, e.g., "The Alcohol Textbook" (Editors K. Jacques, T.P. Lyons and D.R. Kelsall, Nottingham University Press, United Kingdom 1999).

[0153] A fermentation stimulator can be used in combination with any of the processes described herein to further improve the fermentation process, and in particular, the performance of the fermenting microorganism, such as, rate enhancement and ethanol yield. A "fermentation stimulator" refers to stimulators for growth of the fermenting microorganisms, in particular, yeast. Preferred fermentation stimulators for growth include vitamins and minerals. Examples of vitamins include multivitamins, biotin, pantothenate, nicotinic acid, meso-inositol, thiamine, pyridoxine, para-aminobenzoic acid, folic acid, riboflavin, and Vitamins A, B, C, D, and E. See, for example, Alfenore et al., Improving ethanol production and viability of *Saccharomyces cerevisiae* by a vitamin feeding strategy during fed-batch process, Springer-Verlag (2002). Examples of minerals include minerals and mineral salts that can supply nutrients comprising P, K, Mg, S, Ca, Fe, Zn, Mn, and Cu.

[0154] Fermentation products: A fermentation product can be any substance derived from the fermentation. The fermentation product can be, without limitation, an alcohol (e.g., arabinitol, n-butanol, isobutanol, ethanol, glycerol, methanol, ethylene glycol, 1,3-propanediol [propylene glycol], butanediol, glycerin, sorbitol, and xylitol); an alkane (e.g., pentane, hexane, heptane, octane, nonane, decane, undecane, and dodecane), a cycloalkane (e.g., cyclopentane, cyclohexane, cycloheptane, and cyclooctane), an alkene (e.g., pentene, hexene, heptene, and octene); an amino acid (e.g., aspartic acid, glutamic acid, glycine, lysine, serine, and threonine); a gas (e.g., methane, hydrogen (H_2), carbon dioxide (CO_2), and carbon monoxide (CO)); isoprene; a ketone (e.g., acetone); an organic acid (e.g., acetic acid, acetic acid, adipic acid, ascorbic acid, citric acid, 2,5-diketo-D-gluconic acid, formic acid, fumaric acid, glucaric acid, gluconic acid, glucuronic acid, glutaric acid, 3-hydroxypropionic acid, itaconic acid, lactic acid, malic acid, malonic acid, oxalic acid, oxaloacetic acid, propionic acid, succinic acid, and xylonic acid); and polyketide.

[0155] In one aspect, the fermentation product is an alcohol. The term "alcohol" encompasses a substance that contains one or more hydroxyl moieties. The alcohol can be, but is not limited to, n-butanol, isobutanol, ethanol, methanol, arabinitol, butanediol, ethylene glycol, glycerin, glycerol, 1,3-propanediol, sorbitol, xylitol. See, for example, Gong et al., 1999, Ethanol production from renewable resources, in *Advances in Biochemical Engineering/Biotechnology*, Scheper, T., ed., Springer-Verlag Berlin Heidelberg, Germany, 65: 207-241; Silveira and Jonas, 2002, *Appl. Microbiol. Biotechnol.* 59: 400-408; Nigam and Singh, 1995, *Process Biochemistry* 30(2): 117-124; Ezeji et al., 2003, *World Journal of Microbiology and Biotechnology* 19(6): 595-603.

[0156] In another aspect, the fermentation product is an alkane. The alkane may be an unbranched or a branched alkane. The alkane can be, but is not limited to, pentane, hexane, heptane, octane, nonane, decane, undecane, or dodecane.

[0157] In another aspect, the fermentation product is a cycloalkane. The cycloalkane can be, but is not limited to, cyclopentane, cyclohexane, cycloheptane, or cyclooctane.

[0158] In another aspect, the fermentation product is an alkene. The alkene may be an unbranched or a branched alkene. The alkene can be, but is not limited to, pentene, hexene, heptene, or octene.

Fermentation Broth Formulations or Cell Compositions

[0159] The present invention also relates to a fermentation broth formulation or a cell composition comprising a polypeptide of the present invention. The fermentation broth product further comprises additional ingredients used in the fermentation process, such as, for example, cells (including, the host cells containing the gene encoding the polypeptide of the present invention which are used to produce the polypeptide), cell debris, biomass, fermentation media and/or fermentation products. In some embodiments, the composition is a cell-killed whole broth containing organic acid(s), killed cells and/or cell debris, and culture medium.

[0160] The term "fermentation broth" refers to a preparation produced by cellular fermentation that undergoes no or minimal recovery and/or purification. For example, fermentation broths are produced when microbial cultures are grown

to saturation, incubated under carbon-limiting conditions to allow protein synthesis (e.g., expression of enzymes by host cells) and secretion into cell culture medium. The fermentation broth can contain unfractionated or fractionated contents of the fermentation materials derived at the end of the fermentation. Typically, the fermentation broth is unfractionated and comprises the spent culture medium and cell debris present after the microbial cells (e.g., filamentous fungal cells) are removed, e.g., by centrifugation. In some embodiments, the fermentation broth contains spent cell culture medium, extracellular enzymes, and viable and/or nonviable microbial cells.

[0161] In an aspect, the fermentation broth formulation and cell compositions comprise a first organic acid component comprising at least one 1-5 carbon organic acid and/or a salt thereof and a second organic acid component comprising at least one 6 or more carbon organic acid and/or a salt thereof. In a specific embodiment, the first organic acid component is acetic acid, formic acid, propionic acid, a salt thereof, or a mixture of two or more of the foregoing and the second organic acid component is benzoic acid, cyclohexanecarboxylic acid, 4-methylvaleric acid, phenylacetic acid, a salt thereof, or a mixture of two or more of the foregoing.

[0162] In one aspect, the composition contains an organic acid(s), and optionally further contains killed cells and/or cell debris. In one embodiment, the killed cells and/or cell debris are removed from a cell-killed whole broth to provide a composition that is free of these components.

[0163] The fermentation broth formulations or cell compositions may further comprise a preservative and/or anti-microbial (e.g., bacteriostatic) agent, including, but not limited to, sorbitol, sodium chloride, potassium sorbate, and others known in the art.

[0164] The fermentation broth formulations or cell compositions may further comprise multiple enzymatic activities, such as one or more (e.g., several) enzymes selected from the group consisting of a cellulase, a hemicellulase, a cellulose inducible protein (CIP), an esterase, an expansin, a laccase, a lignolytic enzyme, a pectinase, a peroxidase, a protease, and a swollenin. The fermentation broth formulations or cell compositions may also comprise one or more (e.g., several) enzymes selected from the group consisting of a hydrolase, an isomerase, a ligase, a lyase, an oxidoreductase, or a transferase, e.g., an alpha-galactosidase, alpha-glucosidase, aminopeptidase, amylase, beta-galactosidase, beta-glucosidase, beta-xylosidase, carbohydrase, carboxypeptidase, catalase, cellobiohydrolase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, endoglucanase, esterase, glucoamylase, invertase, laccase, lipase, mannosidase, mutanase, oxidase, pectinolytic enzyme, peroxidase, phytase, polyphenoloxidase, proteolytic enzyme, ribonuclease, transglutaminase, or xylanase.

[0165] The cell-killed whole broth or composition may contain the unfractionated contents of the fermentation materials derived at the end of the fermentation. Typically, the cell-killed whole broth or composition contains the spent culture medium and cell debris present after the microbial cells (e.g., filamentous fungal cells) are grown to saturation, incubated under carbon-limiting conditions to allow protein synthesis (e.g., expression of cellulase and/or glucosidase enzyme(s)). In some embodiments, the cell-killed whole broth or composition contains the spent cell culture medium, extracellular enzymes, and killed filamentous fungal cells. In some embodiments, the microbial cells present in the cell-killed whole broth or composition can be permeabilized and/or lysed using methods known in the art.

[0166] A whole broth or cell composition as described herein is typically a liquid, but may contain insoluble components, such as killed cells, cell debris, culture media components, and/or insoluble enzyme(s). In some embodiments, insoluble components may be removed to provide a clarified liquid composition.

[0167] The whole broth formulations and cell compositions of the present invention may be produced by a method described in WO 90/15861 or WO 2010/096673.

[0168] Examples are given below of uses of the compositions of the present invention. The dosage of the composition and other conditions under which the composition is used may be determined on the basis of methods known in the art.

Enzyme Compositions

[0169] The compositions may be prepared in accordance with methods known in the art and may be in the form of a liquid or a dry composition. The compositions may be stabilized in accordance with methods known in the art.

[0170] In yet further aspects of the disclosure, the enzymes of the composition of present invention may be a protease derived from *Bacillus amyloliquefaciens*, a triacylglycerol lipase with a phospho-lipase activity derived from *Thermomyces lanuginosus*, a triacylglycerol lipase derived from *Humicola insolens*, a triacylglycerol lipase derived from *Thermomyces lanuginosus*, NZ81243 - a multicomponent enzyme commercially available as Novozym 81243 with pectate lyase, xylanase and cellulase activities, a beta-glucanase derived from *Aspergillus aculeatus*, an alpha-amylase derived from *Rhizomucor pusillus* and/or an endo-mannosidase derived from *Bacillus bogoriensis*.

[0171] Examples are given below of uses of the compositions of the present invention. The dosage of the composition and other conditions under which the composition is used may be determined on the basis of methods known in the art.

MATERIALS AND METHODS

1. Substrate preparation

[0172] Unsorted MSW is basically made up of two solid fractions: 1) Inorganic non-degradables consisting of plastic, glass, metal etc. and 2) Degradable organics consisting of vegetable, animal waste, food waste, paper, cardboard etc. As an example the latter part typically makes up approximately 65-70% of the incoming waste in Denmark (for a detailed composition analysis see Riber et al. 2009, Waste Management 29, p. 1251-1257).

The scope of the experiments described below was to optimize the solubilization of the degradable organic parts of MSW. Therefore, we choose to use a model waste that reflected the composition of degradable organics in MSW as described by Riber et al. 2009. The model waste consisted of 3 fractions based on fresh products supplemented with water:

- 40.1% Vegetable origin. Mix of fresh vegetables (onions, cabbage, carrots, cucumber etc.), cereals (oatmeals, corn flakes etc.), bread, cake, flowers, boiled rice, boiled pasta fruit, ketchup etc.
- 11.3% Animal origin. Mix of pâté, sausage, hotwings, spareribs, crude meats from chicken, pork and beef etc.
- 36.2% Cellulose origin. Mix of newsprints, office paper, magazines, cardboard, juice cartons, kitchen tissue, cotton, wood, textiles etc.
- Water to obtain a consistency of the model waste suitable in the assays (described later).

[0173] A total of 3 different batches were used prepared and used for the experiments. Batch 1 and 2 was prepared by REnescience (DONG A/S). Batch 3 was prepared in our own laboratories. The composition of the model waste is given in the table below:

Total solids/dry matter (TS)	25.2-28.3%
<i>TS composition:</i>	
Klason lignin	12%
Cellulose	29-32%
Xylan	10%
Arabinan	1%
Galactan	1.7%
Mannnan	4%
Fats	7%
Protein	7-8%
Starch	3-10%
Ash	15%

2. Assay for enzymatic solubilization of model waste in bench scale.

[0174] If not otherwise stated, experiments were carried out on a 20g scale. A standard assay for enzymatic solubilization of model waste was used as follows:

1. 50 ml centrifuge tubes + lid were weighed.
2. Model waste was added to the tubes (total of 1.5 g TS).
3. 50mM citric acid buffer (pH 5) (app. 14.7 ml) and enzymes was added (total weight = 20 g and 7.5% TS) and tubes was vigorously shaken.
4. Tubes were incubated on a Stuart Rotator SB3 at 12 rpm in ovens at 50°C for 24 hours.
5. After incubation, 0.4ml 10% proxel was added to kill lactic acid bacteria and other microorganisms.
6. The tubes were centrifuged 10min at 2090xg.
7. A new set of 50ml tubes were weighed but without lids, marked as supernatant.
8. Supernatant was poured in the tubes and the tubes+ sup was weighed.
9. Approx. 2x 1,5 ml of supernatant was removed and poured in eppendorf tubes for analysis. 50ml tubes with supernatant were weighed again.

11. The first tube + pellet were weighed again.
12. Eppendorf tubes with supernatant were stored in freezer.
13. TS of the supernatant and pellet were determined by drying the tubes at 50°C. The tubes was weighed and then dried at 105°C and weighed again.

[0175] Analysis: Mass balances based on weight were made before and after incubation. If more than 5% of the substrate had been lost samples were discarded. The ratio of the dry matter content in the supernatant and pellet following centrifugation was calculated for all samples. If needed, samples that had been frozen were analyzed for sugar content, acetic acid and lactate on HPLC.

EXAMPLES

Example 1.

Screening of individual enzymes and selection of enzyme candidates for blending.

[0176] Initially, a very broad range of enzymes (>50; Novozymes A/S, Bagsværd, Denmark) were screened for investigating the potential of improving the hydrolysis of the model waste. The tested enzymes included alpha-amylases, gluco-amylase, pullulanase, proteases, lipases, cellulases, xylanases, pectinases and beta-glucanases.

[0177] The described standard assay was used. Control vials were added CBC (CELLIC® Ctec3; Novozymes A/S, Bagsværd, Denmark) in an amount of 2.4%/TS (product to dry matter). In test vials part of CBC was replaced with other enzymes on a protein:protein basis in different ratios ranging from 1-50%. Density of CBC and different products was taken into account. Furthermore, blanks with substrate and buffer but no enzymes were prepared. All tests were carried out in at least duplicates.

[0178] The primary success criteria for an improved hydrolysis were defined as an increase in soluble total solids (TS in supernatant). The ratio of TS-solubilisation during the screening procedure was in general around 25-28% for samples with CBC but variations were seen. The TS-solubilisation of tubes without enzymes was consistent around 10-11%. Candidates were selected for further testing:

- **B.a protease (SEQ ID NO: 1):** A protease derived from *Bacillus amyloliquefaciens* that gave up to 5% increase in TS-solubilization at 30% replacement of CBC.
- **T.l pholip (SEQ ID NO: 2):** A triacylglycerol lipase with phospho-lipase activity derived from *Thermomyces lanuginosus* that gave up to 4.4-8.5% increase in TS-solubilization at 2-20% replacement of CBC.
- **H.i trilip (H.i trilip):** A triacylglycerol lipase derived from *Humicola insolens* that gave up to 4-7% increase in TS-solubilization at 5-20% replacement of CBC.
- **T.l trilip (SEQ ID NO: 3):** A triacylglycerol lipase derived from *Thermomyces lanuginosus* that gave up to 10-15% increase in TS-solubilization at 1-10% replacement of CBC.
- **NZ81243** A multicomponent enzyme commercially available as Novozym 81243 with pectate lyase, xylanase and cellulase activities. Gave up to 4% increase in TS solubilization at 5-20% replacement of CBC. 2:1
- **A.a BG: (SEQ ID NO: 4):** A beta-glucanase derived from *Aspergillus aculeatus*. Contains side activities (cellulase, xylanases, pectinase). Gave up to 6.2-8.2% increase in TS solubilization at 20-40% replacement of CBC. Gave up to 16% in TS solubilization at 30% replacement of CBC when mixed with NZ81243 in a 2:1 ratio.
- **R.p Alam (SEQ ID NO: 5):** A alpha-amylase derived from *Rhizomucor pusillus* that improve glucose yields in the supernatant with up to 20% at 5-20% replacement of CBC.
- **B.b Enma:** An endo-mannosidase derived from *Bacillus bogoriensis*. Gave up to 6-10% increase in TS solubilization at 2.5-10% replacement of CBC.

Example 2.

Designing of optimized enzymes mix by blending of selected candidates

[0179] Statistical experiments were setup to find the optimal ratio between CBC and the selected enzymes candidates in a multicomponent enzymes blend. Based on the screening experiments we decided to use two different templates that specified the enzymes ratios to be used:

For both templates it was decided to use 0-20% *B.a protease* (protease) and 0-10% lipase. This was supplemented with either a) 0-30% *A.a BG* (beta-glucanase) +/- NZ81243 (pectate lyase) or b) 0-20% *B.b Enma* (endo-mannosidase) or 0-20% *R.p Alam* (alpha-amylase). A total of 6 blending experiments were performed.

[0180] Experiment a.1-a.3. The initial three experiments were carried out to select the most suitable lipase when

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combined with 0-20% *B.a protease* and 0-30% *A.a BG*. The templates were as follows:

Blend template a.1

Enzyme Name	Ratio of total enzyme protein:
<i>B.a protease</i>	0-20%
<i>T.l pholip</i>	0-10%
<i>A.a BG</i>	0-30%
CBC	Supplement to 100%

Blend template a.2

Enzyme Name	Ratio of total enzyme protein:
<i>B.a protease</i>	0-20%
<i>T.l trilip</i>	0-10%
<i>A.a BG</i>	0-30%
CBC	Supplement to 100%

Blend template a.3

Enzyme Name	Ratio of total enzyme protein:
<i>B.a protease</i>	0-20%
<i>H.i trilip</i>	0-10%
<i>A.a BG</i>	0-30%
CBC	Supplement to 100%

[0181] Statistical discovery software (Design Of Experiments, DOE) by JMP® was used to design the dosing in the experiment (see table below). As in Example 1, the enzymes concentration in control vials (vial 17 and 39) was 2.4% CBC/TS. In test vials part of CBC was replaced with other enzymes on a protein:protein basis as stated in the table below. Each test blend was carried out in duplicates.

Enzymes dosing in tubes. Used for template a.1, a. 2, a.3, a.4, a.5.

Tube	CBC	<i>B.a protease</i>	<i>T.l pholip</i> or <i>T.l trilip</i> or <i>H.i trilip</i>	<i>A.a BG</i>
#	Ratio (% of enzyme protein)			
12	0,4	0,2	0,1	0,3
22	0,4	0,2	0,1	0,3
6	0,5	0,2	0	0,3
19	0,5	0,1	0,1	0,3
21	0,5	0,1	0,1	0,3
25	0,5	0,2	0	0,3
10	0,55	0,2	0,1	0,15
26	0,55	0,2	0,1	0,15
9	0,6	0	0,1	0,3
13	0,6	0,1	0	0,3
29	0,6	0,1	0	0,3
36	0,6	0	0,1	0,3
3	0,65	0	0,05	0,3
4	0,65	0,2	0	0,15

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(continued)

Tube	CBC	<i>B.a protease</i>	<i>T.l pholip</i> or <i>T.l trilip</i> or <i>H.i trilip</i>	<i>A.a BG</i>
#	Ratio (% of enzyme protein)			
28	0,65	0	0,05	0,3
33	0,65	0,2	0	0,15
2	0,7	0,2	0,1	0
5	0,7	0,1	0,05	0,15
15	0,7	0	0	0,3
23	0,7	0,1	0,05	0,15
24	0,7	0,2	0,1	0
35	0,7	0	0	0,3
7	0,75	0,2	0,05	0
16	0,75	0	0,1	0,15
30	0,75	0	0,1	0,15
31	0,75	0,2	0,05	0
8	0,8	0,2	0	0
11	0,8	0,1	0,1	0
37	0,8	0,2	0	0
38	0,8	0,1	0,1	0
14	0,85	0	0	0,15
34	0,85	0	0	0,15
18	0,9	0	0,1	0
20	0,9	0,1	0	0
27	0,9	0	0,1	0
32	0,9	0,1	0	0
1	0,95	0	0,05	0
40	0,95	0	0,05	0
17	1	0	0	0
39	1	0	0	0

[0182] The highlights of test a.1, a.2 and a.3 are given in the tables below:

Results highlights blend a.1	
Enzyme components	<i>B.a protease, T.l pholip, A.a BG, CBC</i>
Result control (TS solubilization)	25.8%
1. best blend (TS solubilization)	32.8%
Improvement	27.1%
Best ratio between enzymes	10:5:15:70
2. best blend (TS solubilization)	30,8%
Improvement	19.4%
Best ratio between enzymes	10:10:0:80
Best ratio according to model	13:7:11:69
Results highlights blend a.2	
Enzyme components	<i>B.a protease, T.l trilip, A.a BG, CBC</i>
Result control (TS solubilization)	28.1 %
1. best blend (TS solubilization)	34.7%

(continued)

Results highlights blend a.2

Enzyme components	<i>B.a protease, T.I trilip, A.a BG, CBC</i>
Improvement	23.5%
Best ratio between enzymes	20:10:30:40
2. best blend (TS solubilization)	33.4%
Improvement	18.9%
Best ratio between enzymes	10:10:0:80

Results highlights blend a.3

Enzyme components	<i>B.a protease, H.i trilip, A.a BG, CBC</i>
Result control (TS solubilization)	26.1%
1. best blend (TS solubilization)	32.5%
Improvement	24.5%
Best ratio between enzymes	10:5:15:70
2. best blend (TS solubilization)	31.9%
Improvement	22.2%
Best ratio between enzymes	20:5:0:80

[0183] As shown, the best improvement over CBC in TS-solubilization was found in experiment a.1 with a 27.1% increase with a ratio of 10:5:15:70 for *B.a protease: T.I pholip:A.a BG:CBC*. Interestingly, the TS-solubilization in the individual vials showed significant deviations ranging from a slight negative impact by the enzymes mix to a pronounced boost of +30.4% in tube number 23. As written in Example 1, the individual improvements of *B.a protease, T.I pholip* and *A.a BG* was up to 5%, 8.5% and 8.2% respectively. Multiplication of the individual effects only adds up to 21.7%. Thus, a synergistic effect was clearly obtained in experiment a.1 when mixing the enzymes.

[0184] Clear improvements in TS solubilization was also seen in experiment a.2 (up to 23.5% with a combination of *B.a protease.T.I trilip:A.a BG:CBC* in a ratio of 20:10:30:40) and a.3 (up to 24.5% with a combination of *B.a protease:H.i trilip:A.a BG:CBC* in a ratio of 10:5:15:70). It was however decided to continue with *T.I pholip* in the following experiments.

[0185] Experiment a.4. This experiment was used to decide whether *A.a BG* should be supplemented with *NZ81243*. The initial screening (Example 1) had shown a boost in TS solubilization when the two enzymes was combined.

Blend template a.4

Enzyme Name	Ratio of total enzyme protein:
<i>B.a protease</i>	0-20%
<i>T.I pholip</i>	0-10%
<i>A.a BG + NZ81243</i> (2:1 mix)	0-30%
CBC	Supplement to 100%

[0186] As shown in the table below, TS-solubilization could be increased to 22.1%. However, this was a smaller improvement than in experiment a.1 where *A.a BG* was not supplemented with *NZ81243*.

Results highlights blend a.4

Enzyme components	<i>B.a protease, T.I pholip, A.a BG+NZ81243, CBC</i>
Result control (TS solubilization)	26.6%
1. best blend (TS solubilization)	32.5%
Improvement	22.1%

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(continued)

Results highlights blend a.4

Enzyme components	<i>B.a protease</i> , <i>T.l pholip</i> , <i>A.a BG</i> + <i>NZ81243</i> , CBC
Best ratio between enzymes	0:5:30:65
2. best blend (TS solubilization)	32.3%
Improvement	21.4%
Best ratio between enzymes	20:5:0:75

[0187] Experiment b.1. and b.2. This experiment was used to decide wheter the alpha amylase *R.p Alam* or endo-mannosidase *B.b Enma* where a better "match" for the protease *B.a protease* and the lipase *T.l pholip* than *A.a BG*. The following blend and dosing templates were used:

Blend template b.1

Enzyme Name	Ratio of total enzyme protein:
<i>B.a protease</i>	0-20%
<i>T.l pholip</i>	0-10%
<i>B.b Enma</i>	0-10%
CBC	Supplement to 100%

Blend template b.2

Enzyme Name	Ratio of total enzyme protein:
<i>B.a protease</i>	0-20%
<i>T.l pholip</i>	0-10%
<i>R.p Alam</i>	0-10%
CBC	Supplement to 100%

Enzymes dosing in tubes. Used for template b.1, b.2.

Tube	CBC	<i>B.a protease</i>	<i>T.l pholip</i>	<i>B.b Enma</i> or <i>R.p Alam</i>
#	Ratio (% of enzyme protein)			
15	0,6	0,2	0,1	0,1
37	0,6	0,2	0,1	0,1
10	0,65	0,2	0,05	0,1
17	0,65	0,2	0,1	0,05
27	0,65	0,2	0,1	0,05
40	0,65	0,2	0,05	0,1
3	0,7	0,1	0,1	0,1
5	0,7	0,2	0	0,1
8	0,7	0,2	0,1	0
29	0,7	0,2	0	0,1
30	0,7	0,1	0,1	0,1
35	0,7	0,2	0,1	0
16	0,75	0,2	0,05	0
20	0,75	0,2	0	0,05
26	0,75	0,2	0,05	0
28	0,75	0,2	0	0,05

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(continued)

Tube	CBC	<i>B.a protease</i>	<i>T.l pholip</i>	<i>B.b Enma</i> or <i>R.p Alam</i>
#	Ratio (% of enzyme protein)			
13	0,8	0,1	0,1	0
14	0,8	0	0,1	0,1
18	0,8	0,1	0,05	0,05
19	0,8	0,2	0	0
31	0,8	0,2	0	0
33	0,8	0,1	0,1	0
34	0,8	0	0,1	0,1
38	0,8	0,1	0,05	0,05
4	0,85	0	0,05	0,1
12	0,85	0	0,1	0,05
32	0,85	0	0,05	0,1
39	0,85	0	0,1	0,05
2	0,9	0	0	0,1
6	0,9	0	0,1	0
9	0,9	0,1	0	0
21	0,9	0	0,1	0
24	0,9	0	0	0,1
25	0,9	0,1	0	0
1	0,95	0	0	0,05
7	0,95	0	0,05	0
22	0,95	0	0,05	0
23	0,95	0	0	0,05
11	1	0	0	0
36	1	0	0	0

Results highlights blend b.1

Enzyme components	<i>B.a protease</i> , <i>T.l pholip</i> , <i>B.b Enma</i> , CBC
Result control (TS solubilization)	27.4%
1. best blend (TS solubilization)	34.0%
Improvement	24.1%
Best ratio between enzymes	20:0:30:50
2. best blend (TS solubilization)	33.1%
Improvement	20.8%
Best ratio between enzymes	20:5:10:65

Results highlights blend b.2

Enzyme components	<i>B.a protease</i> , <i>T.l pholip</i> , <i>R.p Alam</i> , CBC
Result control (TS solubilization)	25.7%
1. best blend (TS solubilization)	30.7%
Improvement	19.4%
Best ratio between enzymes	0:5:30:60
2. best blend (TS solubilization)	30.3%
Improvement	17.9%

(continued)

Results highlights blend b.2

Enzyme components	<i>B.a protease, T.l pholip, R.p Alam, CBC</i>
Best ratio between enzymes	20:5:0:75

[0188] In both blending experiments an improved performance in TS-solubilization was found when compared to CBC, but all test were inferior to the blend ratio found in experiment a.1 where *B.a protease, T.l pholip, A.a BG*, where mixed with CBC in a ratio of 10:5:15:70.

[0189] Based on these observations it was decided to use this enzymes combination in the further development.

Example 3.**Testing the selected enzymes blend and CELLIC® Ctec3 in free fall experiments at elevated dry matter concentration.**

[0190] An experiment was carried out to test the efficiency of the selected enzymes blend at a dry matter concentration that was higher than during the screening and blending experiments. However, due to a high viscosity of model waste, mixing is not optimal in 50ml tubes when experiments are carried at dry matter concentrations above 7.5%. Instead, experiments were performed in 100ml Kautex bottles. Model waste was mixed with water to a volume of 50ml and at TS concentration of 7.5% and 15%. CBC and the selected blend (*B.a protease: T.l pholip:A.a BG*:CBC in ratio of 10:5:15:70) were added in an amount of 2.4%/TS (product to dry matter CBC). Tubes were incubated for 24 hours at 50°C in the tumbler-reactor. Results are shown in Figure 1.

[0191] TS solubilization of original biomass in control tubes (no enzymes) were 10.9% and 3.2% at 7.5% and 15%, respectively. Addition of CBC improved TS-solubilization to 29.6% and 25.8. Supplementing CBC with the other enzymes improved solubilization further to 37.1 and 30.7%. This corresponds to a relative improvement of 25.7% (7.5% dry matter) and 18.9% (15% dry matter), when comparing the blend with CBC. The numbers obtained at 7.5% dry matter confirms the improvement seen in 20g scale (50 ml tubes), which was up to 27.3%.

Example 4. Comparable dose-response experiments with CBC and the enzymes blend.

[0192] Experiments were performed in 100ml Kautex bottles. Model waste was mixed with water to a volume at 50ml and at TS concentration of 7.5%. CBC and the selected blend (*B.a protease: T.l pholip:A.a BG*:CBC in ratio of 10:5:15:70) were added in amounts corresponding to 0%, 25%, 50%, 75%, 100% and 200% of the concentration that has been used as default during the previous experiments (2.4% enzymes protein/TS). Bottles were incubated on a Stuart Rotator SB3 and placed in a 50°C oven for 24 hours.

[0193] A significant improvement in TS-solubilization was seen at all applied enzyme concentrations, when comparing the blend with CBC. The TS-solubilization at default settings (2.4% CBC/TS) was around 25%. This was obtained with only approximately 0.9% of the blend, which corresponds to a lowering in enzyme dosage of approximately 2.5 to 2.7 times (See figure 2). At the same time we found a clear increase in hydrolysis and fermentation products such as glucose, xylose, lactic acid (Figure 3, and Figure 5). This is a surprise since 15% of CBC (cellulase and xylanase activities) was replaced with the lipase and protease.

Example 5. Omitting individual components from blend

[0194] An experiment was carried out to test the relevance of the individual enzymes in the optimized blend (*B.a protease: T.l pholip:A.a BG*:CBC in ratio of 10:5:15:70). The setup of control vials was as described in Example 1 (2.4% CELLIC® Ctec, 7.5% TS, 20 gram scale). In test vials the optimized blend was applied, including all enzymes. At the same time test vials were made where either *B.a protease, T.l pholip* or *A.a BG* had been excluded from the blend.

[0195] The effect on TS-solubilization is illustrated in figure 4 and clearly shows that removing any of the individual enzymes resulted in a lower TS-solubilization, when compared to vials with all enzymes. However, when only using two (2) of the selected enzymes to supplement CBC (*T.l pholip+A.a BG, B.a protease+A.a BG, B.a protease+T.l pholip*) we still observed an improved TS-solubilization, when compared to vials with only CBC, even though total enzymes protein concentration had been lowered by removing individual enzymes.

SEQUENCE LISTING

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			35					40					45				
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15																	
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					85					90					95		
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40					165					170					175		
	Trp	Val	Gly	Asn	Tyr	Ser	Phe	Asp	Gly	Ile	Arg	Ile	Asp	Thr	Val	Lys	
				180					185					190			
45																	
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50																	
55																	

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	195	200	205
5	Phe Ala Thr Gly Glu Val 210	Phe Asn Gly Asp Pro 215	Ala Tyr Val Gly Pro 220
10	Tyr Gln Lys Tyr Leu Pro 225	Ser Leu Ile Asn Tyr 230	Pro Met Tyr Tyr Ala 235
15	Leu Asn Asp Val Phe Val 245	Ser Lys Ser Lys Gly Phe 250	Ser Arg Ile Ser 255
20	Glu Met Leu Gly Ser Asn Arg 260	Asn Ala Phe Glu Asp Thr 265	Ser Val Leu 270
25	Thr Thr Phe Val Asp Asn His 275	Asp Asn Pro Arg Phe 280	Leu Asn Ser Gln 285
30	Ser Asp Lys Ala Leu Phe 290	Lys Asn Ala Leu Thr 295	Tyr Val Leu Leu Gly 300
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40	Gly Ala Asp Pro Ala Asn Arg 325	Glu Val Leu Trp Thr Thr 330	Asn Tyr Asp 335
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75	Ala Ile Phe Thr Ser Ala Thr 435	Gly Gly Thr Thr Thr Thr 440	Ala Thr Pro 445

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					485					490					495	
15	Ile	Tyr	Leu	Val	Gly	Ser	Ile	Ser	Gln	Leu	Gly	Asp	Trp	Glu	Thr	Ser
				500					505					510		
20	Asp	Gly	Ile	Ala	Leu	Ser	Ala	Asp	Lys	Tyr	Thr	Ser	Ser	Asp	Pro	Leu
			515					520					525			
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30	Phe	Ile	Arg	Ile	Glu	Ser	Asp	Asp	Ser	Val	Glu	Trp	Glu	Ser	Asp	Pro
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	1				5					10					15	
60	Gly	Asn	Pro	Phe	Val	Met	Arg	Gly	Ile	Asn	His	Gly	His	Ala	Trp	Tyr
				20					25					30		
65	Lys	Asp	Gln	Ala	Thr	Thr	Ala	Ile	Glu	Gly	Ile	Ala	Asn	Thr	Gly	Ala
			35					40					45			
70	Asn	Thr	Val	Arg	Ile	Val	Leu	Ser	Asp	Gly	Gly	Gln	Trp	Thr	Lys	Asp
	50						55					60				
75	Asp	Ile	His	Thr	Val	Arg	Asn	Leu	Ile	Ser	Leu	Ala	Glu	Asp	Asn	His
	65					70					75				80	

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	Leu	Val	Ala	Val	Leu	Glu	Val	His	Asp	Ala	Thr	Gly	Tyr	Asp	Ser	Ile	
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5	Ala	Ser	Leu	Asn	Arg	Ala	Val	Asp	Tyr	Trp	Ile	Glu	Met	Arg	Ser	Ala	
				100					105						110		
10	Leu	Ile	Gly	Lys	Glu	Asp	Thr	Val	Ile	Ile	Asn	Ile	Ala	Asn	Glu	Trp	
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15	Phe	Gly	Ser	Trp	Glu	Gly	Asp	Ala	Trp	Ala	Asp	Gly	Tyr	Lys	Gln	Ala	
		130					135					140					
20	Ile	Pro	Arg	Leu	Arg	Asn	Ala	Gly	Leu	Asn	His	Thr	Leu	Met	Val	Asp	
	145					150					155					160	
25	Ala	Ala	Gly	Trp	Gly	Gln	Phe	Pro	Gln	Ser	Ile	His	Asp	Tyr	Gly	Arg	
					165					170					175		
30	Glu	Val	Phe	Asn	Ala	Asp	Pro	Gln	Arg	Asn	Thr	Met	Phe	Ser	Ile	His	
			180						185					190			
35	Met	Tyr	Glu	Tyr	Ala	Gly	Gly	Asn	Ala	Ser	Gln	Val	Arg	Thr	Asn	Ile	
			195					200					205				
40	Asp	Arg	Val	Leu	Asn	Gln	Asp	Leu	Ala	Leu	Val	Ile	Gly	Glu	Phe	Gly	
		210				215						220					
45	His	Arg	His	Thr	Asn	Gly	Asp	Val	Asp	Glu	Ala	Thr	Ile	Met	Ser	Tyr	
	225					230					235					240	
50	Ser	Glu	Gln	Arg	Gly	Val	Gly	Trp	Leu	Ala	Trp	Ser	Trp	Lys	Gly	Asn	
				245					250						255		
55	Gly	Pro	Glu	Trp	Glu	Tyr	Leu	Asp	Leu	Ser	Asn	Asp	Trp	Ala	Gly	Asn	
			260					265						270			
60	Asn	Leu	Thr	Ala	Trp	Gly	Asn	Thr	Ile	Val	Asn	Gly	Pro	Tyr	Gly	Leu	
			275					280					285				
65	Arg	Glu	Thr	Ser	Arg	Leu	Ser	Thr	Val	Phe	Thr	Gly	Gly	Gly	Ser	Asp	
		290					295					300					
70	Gly	Gly	Thr	Ser	Pro												
	305																

Claims

1. An enzyme composition for solubilization of Municipal Solid Waste, the enzyme composition comprising a) an *Aspergillus fumigatus* cellobiohydrolase I; (b) an *Aspergillus fumigatus* cellobiohydrolase II; (c) an *Aspergillus fumigatus* beta-glucosidase; and (d) a *Penicillium* sp. GH61 polypeptide having cellulolytic enhancing activity and enzymes selected from (i) a protease; (ii) a lipase and (iii) a beta-glucanase, wherein: the protease (i) is selected from SEQ ID NO: 1 or a protease having at least 90% sequence identity to SEQ ID NO: 1; the lipase (ii) is selected from SEQ ID NO: 2 or a lipase having at least 90% sequence identity to SEQ ID NO: 2; the beta-glucanase is selected from SEQ ID NO: 4 or a beta-glucanase having at least 90% sequence identity to SEQ ID NO: 4.
2. The composition of claim 1, further comprising one or more enzymes selected from (iv) a pectate lyase; (v) a mannanase; and (vi) an amylase.
3. The composition of any of claims 1 to 2, wherein the mannanase (v) is an endo-mannosidase of SEQ ID NO: 6 or an endo-mannosidase having at least 85% sequence identity to SEQ ID NO: 6.
4. The composition of any of claims 2 to 3, wherein the amylase (vi) is an alpha-amylase of SEQ ID NO: 5 or an alpha-amylase having at least 85% sequence identity to SEQ ID NO: 5.
5. The composition of any of claims 1 to 4, wherein the protease is present at a ratio between 0-20% w/w, such as e.g. about 5-15%, or about 10% w/w of the total enzyme protein.
6. The composition of any of claims 1 to 5, wherein the lipase is present at a ratio between 0-10% w/w, such as e.g. about 2.5-7.5%, or about 5% w/w of the total enzyme protein.
7. The composition of any of claims 1 to 6, wherein the beta-glucanase is present at a ratio between 0-30% w/w, such as e.g. 10-20%, or about 15% w/w of the total enzyme protein.
8. The composition of any of claims 2 to 7, wherein the pectate-lyase is present at a ratio between 0-10% w/w, such as e.g. 2.5-7.5%, or about 5% w/w of the total enzyme protein.
9. The composition of any of claims 2 to 8, wherein the mannanase or amylase is present at a ratio between 0-10% w/w, such as e.g. about 2.5-7.5%, or about 5% w/w of the total enzyme protein.
10. The composition of any of claims 1 to 9, wherein a cellulolytic background composition comprises a) a cellobiohydrolase I; (b) a cellobiohydrolase II; (c) a beta-glucosidase; and (d) a GH61 polypeptide having cellulolytic enhancing activity is present in the enzyme composition at a ratio between 40%-99% w/w, such as e.g. between 50%-90% w/w, such as e.g. 60%-80% w/w, such as e.g. 65-75% of the total enzyme protein.
11. The composition of any of claims 1 to 10, wherein the enzyme composition further comprises one or more enzymes selected from a cellulase, an AA9 polypeptide, a hemicellulase, a cellulose inducible protein (CIP) an esterase, an expansin, a ligninolytic enzyme, an oxidoreductase, a pectinase, a protease, and a swollenin.
12. The composition of claim 11, wherein the hemicellulase is one or more enzymes selected from a xylanase, an acetylxylan esterase, a feruloyl esterase, an arabinofuranosidase, a xylosidase, and a glucuronidase.

Patentansprüche

1. Enzymzusammensetzung zur Solubilisierung von kommunalem Festabfall, wobei die Enzymzusammensetzung a) eine *Aspergillus fumigatus*-Cellobiohydrolase I; (b) eine *Aspergillus fumigatus*-Cellobiohydrolase II; (c) eine *Aspergillus fumigatus*-beta-Glucosidase; und (d) ein *Penicillium* sp. GH61-Polypeptid mit cellulolytisch verstärkender Aktivität und Enzyme ausgewählt aus (i) einer Protease; (ii) einer Lipase und (iii) einer beta-Glucanase umfasst, wobei: die Protease (i) ausgewählt ist aus SEQ ID NO: 1 oder einer Protease mit mindestens 90% Sequenzidentität zu SEQ ID NO: 1; die Lipase (ii) ausgewählt ist aus SEQ ID NO: 2 oder einer Lipase mit mindestens 90% Sequenzidentität zu SEQ ID NO: 2;

die beta-Glucanase ausgewählt ist aus SEQ ID NO: 4 oder einer beta-Glucanase mit mindestens 90% Sequenzidentität zu SEQ ID NO: 4.

2. Zusammensetzung nach Anspruch 1, die des Weiteren ein oder mehrere Enzyme umfasst, die ausgewählt sind aus (iv) einer Pektatlyase; (v) einer Mannanase; und (vi) einer Amylase.
3. Zusammensetzung nach einem beliebigen der Ansprüche 1 bis 2, wobei die Mannanase (v) eine endo-Mannosidase von SEQ ID NO: 6 oder eine endo-Mannosidase mit mindestens 85% Sequenzidentität zu SEQ ID NO: 6 ist.
4. Zusammensetzung nach einem beliebigen der Ansprüche 2 bis 3, wobei die Amylase (vi) eine alpha-Amylase von SEQ ID NO: 5 oder eine alpha-Amylase mit mindestens 85% Sequenzidentität zu SEQ ID NO: 5 ist.
5. Zusammensetzung nach einem beliebigen der Ansprüche 1 bis 4, wobei die Protease in einem Verhältnis zwischen 0-20% Gew./Gew., wie z.B. etwa 5-15%, oder etwa 10% Gew./Gew. des gesamten Enzymproteins vorliegt.
6. Zusammensetzung nach einem beliebigen der Ansprüche 1 bis 5, wobei die Lipase in einem Verhältnis zwischen 0-10% Gew./Gew., wie z.B. etwa 2,5-7,5% oder etwa 5% Gew./Gew. des gesamten Enzymproteins vorliegt.
7. Zusammensetzung nach einem beliebigen der Ansprüche 1 bis 6, wobei die beta-Glucanase in einem Verhältnis zwischen 0-30% Gew./Gew., wie z.B. 10-20% oder etwa 15% Gew./Gew. des gesamten Enzymproteins vorliegt.
8. Zusammensetzung nach einem beliebigen der Ansprüche 2 bis 7, wobei die Pektatlyase in einem Verhältnis zwischen 0-10% Gew./Gew., wie z.B. 2,5-7,5% oder etwa 5% Gew./Gew. des gesamten Enzymproteins vorliegt.
9. Zusammensetzung nach einem beliebigen der Ansprüche 2 bis 8, wobei die Mannanase oder Amylase in einem Verhältnis zwischen 0-10% Gew./Gew., wie z.B. etwa 2,5-7,5%, oder etwa 5% Gew./Gew. des gesamten Enzymproteins vorliegt.
10. Zusammensetzung nach einem beliebigen der Ansprüche 1 bis 9, wobei eine cellulolytische Hintergrund-Zusammensetzung umfasst a) eine Cellobiohydrolase I; (b) eine Cellobiohydrolase II; (c) eine beta-Glucosidase; und (d) ein GH61-Polypeptid mit cellulolytisch verstärkender Aktivität in der Enzymzusammensetzung in einem Verhältnis zwischen 40%-99% Gew./Gew., wie z.B. zwischen 50%-90% Gew./Gew., wie z.B. 60%-80% Gew./Gew., wie z.B. 65-75% des gesamten Enzymproteins vorliegt.
11. Zusammensetzung nach einem beliebigen der Ansprüche 1 bis 10, wobei die Enzymzusammensetzung des Weiteren ein oder mehrere Enzyme umfasst, die aus einer Cellulase, einem AA9-Polypeptid, einer Hemicellulase, einem celluloseinduzierbaren Protein (CIP), einer Esterase, einem Expansin, einem ligninolytischen Enzym, einer Oxidoreduktase, einer Pektinase, einer Protease und einem Swollenin ausgewählt sind.
12. Zusammensetzung nach Anspruch 11, wobei die Hemicellulase eines oder mehrere Enzyme ausgewählt aus einer Xylanase, einer Acetylxylan-Esterase, einer Feruloyl-Esterase, einer Arabinofuranosidase, einer Xylosidase und einer Glucuronidase ist.

Revendications

1. Composition enzymatique pour la solubilisation de déchets solides urbains, la composition enzymatique comprenant a) une cellobiohydrolase I d'*Aspergillus fumigatus* ; (b) une cellobiohydrolase II d'*Aspergillus fumigatus* ; (c) une bêta-glucosidase d'*Aspergillus fumigatus* ; et (d) un polypeptide de *Penicillium* sp. GH61 ayant une activité d'amplification cellulolytique, et des enzymes choisies parmi (i) une protéase ; (ii) une lipase et (iii) une bêta-glucanase, dans laquelle :

la protéase (i) est choisie parmi la SEQ ID NO : 1 ou une protéase ayant une identité de séquence d'au moins 90 % avec la SEQ ID NO : 1 ;

la lipase (ii) est choisie parmi la SEQ ID NO : 2 ou une lipase ayant une identité de séquence d'au moins 90 % avec la SEQ ID NO : 2 ;

la bêta-glucanase est choisie parmi la SEQ ID NO : 4 ou une bêta-glucanase ayant une identité de séquence d'au moins 90 % avec la SEQ ID NO : 4.

2. Composition selon la revendication 1, comprenant en outre une ou plusieurs enzymes choisies parmi (iv) une pectate lyase ; (v) une mannanase ; et (vi) une amylase.
3. Composition selon l'une quelconque des revendications 1 à 2, dans laquelle la mannanase (v) est une endo-mannosidase de la SEQ ID NO : 6 ou une endo-mannosidase ayant une identité de séquence d'au moins 85 % avec la SEQ ID NO : 6.
4. Composition selon l'une quelconque des revendications 2 à 3, dans laquelle l'amylase (vi) est une alpha-amylase de la SEQ ID NO : 5 ou une alpha-amylase ayant une identité de séquence d'au moins 85 % avec la SEQ ID NO : 5.
5. Composition selon l'une quelconque des revendications 1 à 4, dans laquelle la protéase est présente en une proportion comprise entre 0 et 20 % en poids/poids, telle que par exemple d'environ 5 à 15 %, ou d'environ 10 % en poids/poids des protéines enzymatiques totales.
6. Composition selon l'une quelconque des revendications 1 à 5, dans laquelle la lipase est présente en une proportion comprise entre 0 et 10 % en poids/poids, telle que par exemple d'environ 2,5 à 7,5 %, ou d'environ 5 % en poids/poids des protéines enzymatiques totales.
7. Composition selon l'une quelconque des revendications 1 à 6, dans laquelle la bêta-glucanase est présente en une proportion comprise entre 0 et 30 % en poids/poids, telle que par exemple 10 à 20 %, ou d'environ 15 % en poids/poids des protéines enzymatiques totales.
8. Composition selon l'une quelconque des revendications 2 à 7, dans laquelle la pectate-lyase est présente en une proportion comprise entre 0 et 10 % en poids/poids, telle que par exemple 2,5 à 7,5 %, ou d'environ 5 % en poids/poids des protéines enzymatiques totales.
9. Composition selon l'une quelconque des revendications 2 à 8, dans laquelle la mannanase ou l'amylase est présente en une proportion comprise entre 0 et 10 % en poids/poids, telle que par exemple d'environ 2,5 à 7,5 %, ou d'environ 5 % en poids/poids des protéines enzymatiques totales.
10. Composition selon l'une quelconque des revendications 1 à 9, dans laquelle une composition d'arrière-plan cellulolytique comprenant a) une cellobiohydrolase I ; (b) une cellobiohydrolase II ; (c) une bêta-glucosidase ; et (d) un polypeptide GH61 ayant une activité d'amplification cellulolytique est présente dans la composition enzymatique en une proportion comprise entre 40 % et 99 % en poids/poids, telle que par exemple entre 50 % et 90 % en poids/poids, telle que par exemple de 60 % à 80 % en poids/poids, telle que par exemple de 65 à 75 % des protéines enzymatiques totales.
11. Composition selon l'une quelconque des revendications 1 à 10, dans laquelle la composition enzymatique comprend en outre une ou plusieurs enzymes choisies parmi une cellulase, un polypeptide AA9, une hémicellulase, une protéine inductible par la cellulose (CIP), une estérase, une expansine, une enzyme ligninolytique, une oxydoréductase, une pectinase, une protéase, et une swollénine.
12. Composition selon la revendication 11, dans laquelle l'hémicellulase est une ou plusieurs enzymes choisies parmi une xylanase, une acétylxylane estérase, une féruloyle estérase, une arabinofuranosidase, une xylosidase, et une glucuronidase.

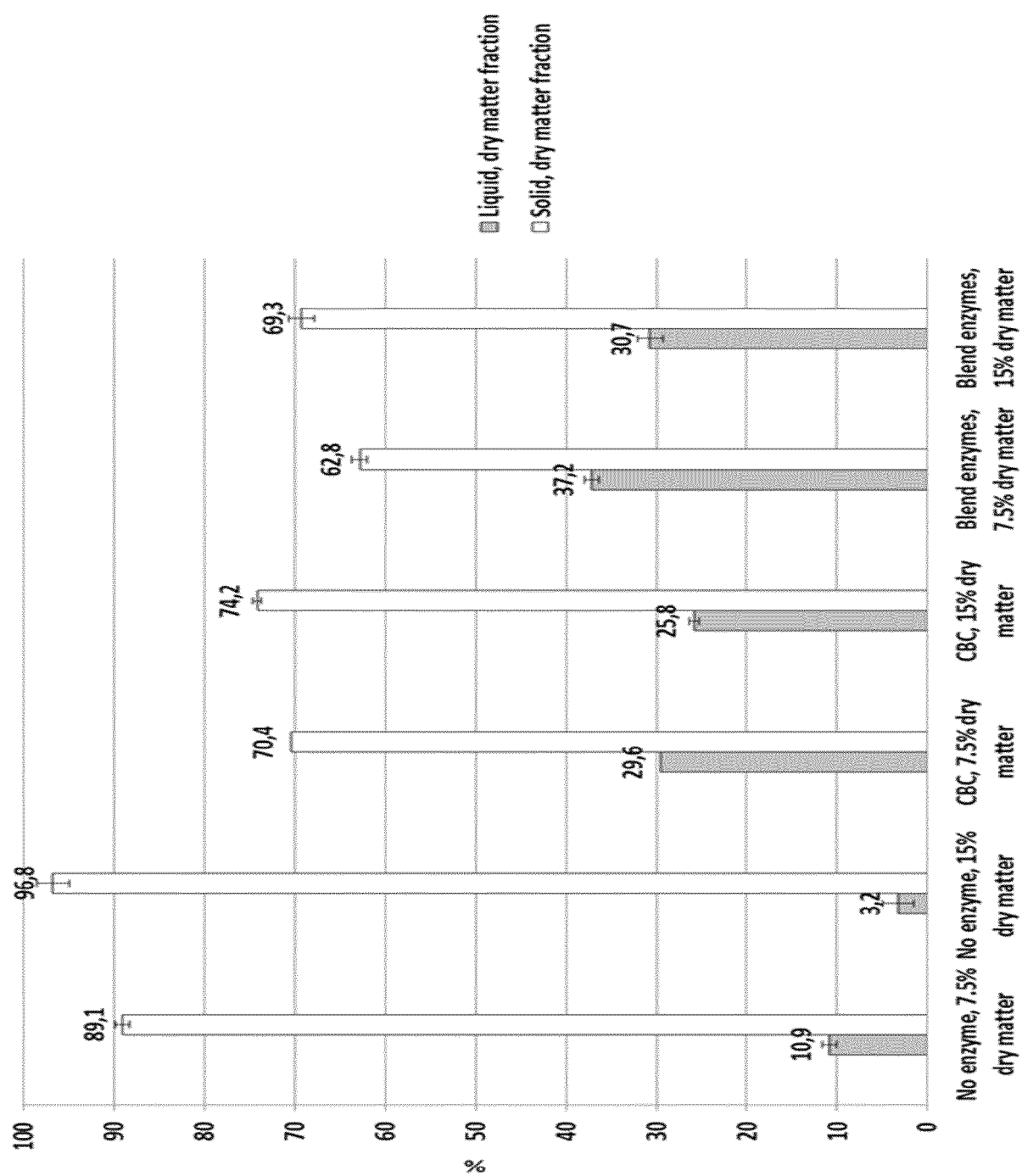


FIG 1

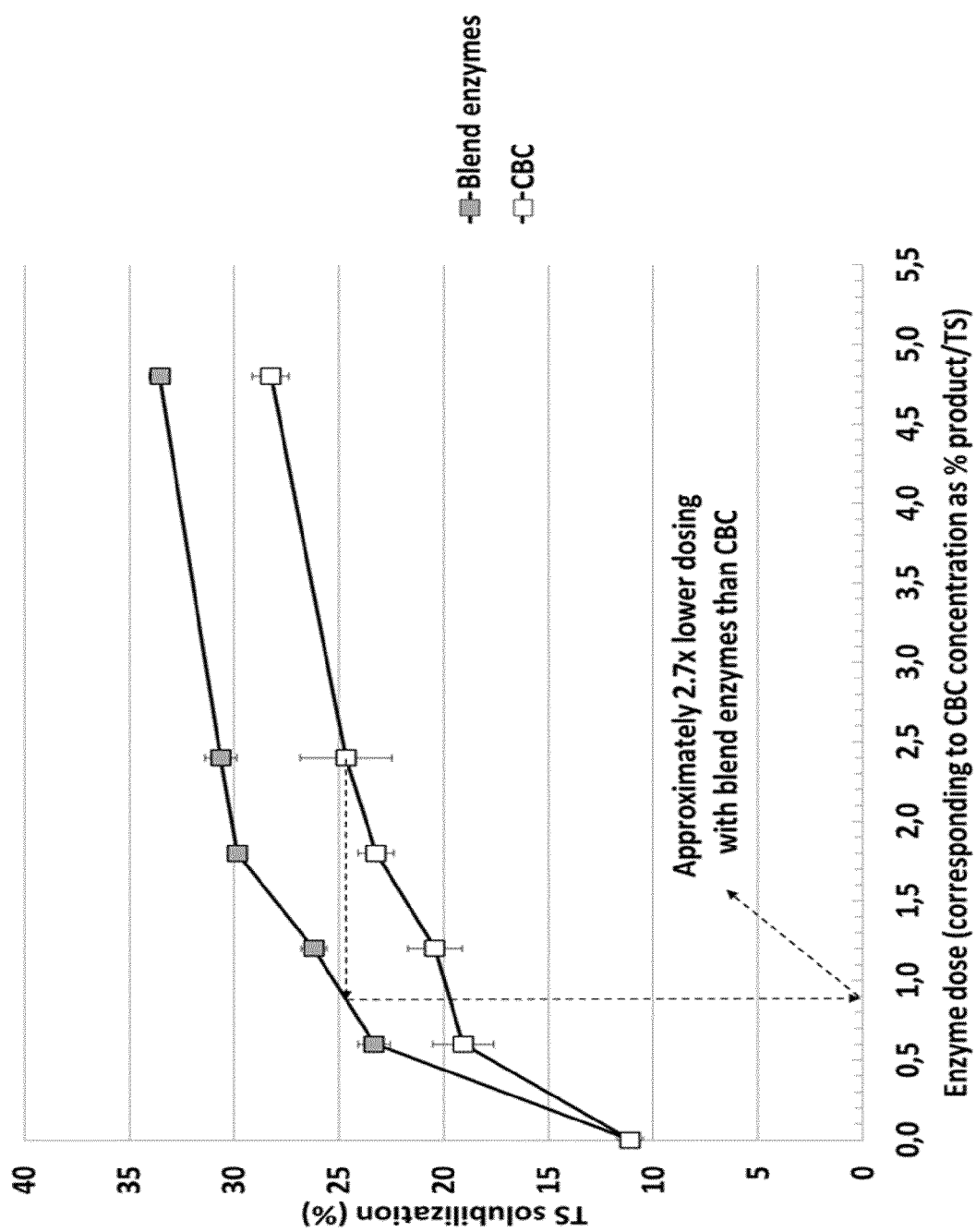


FIG 2

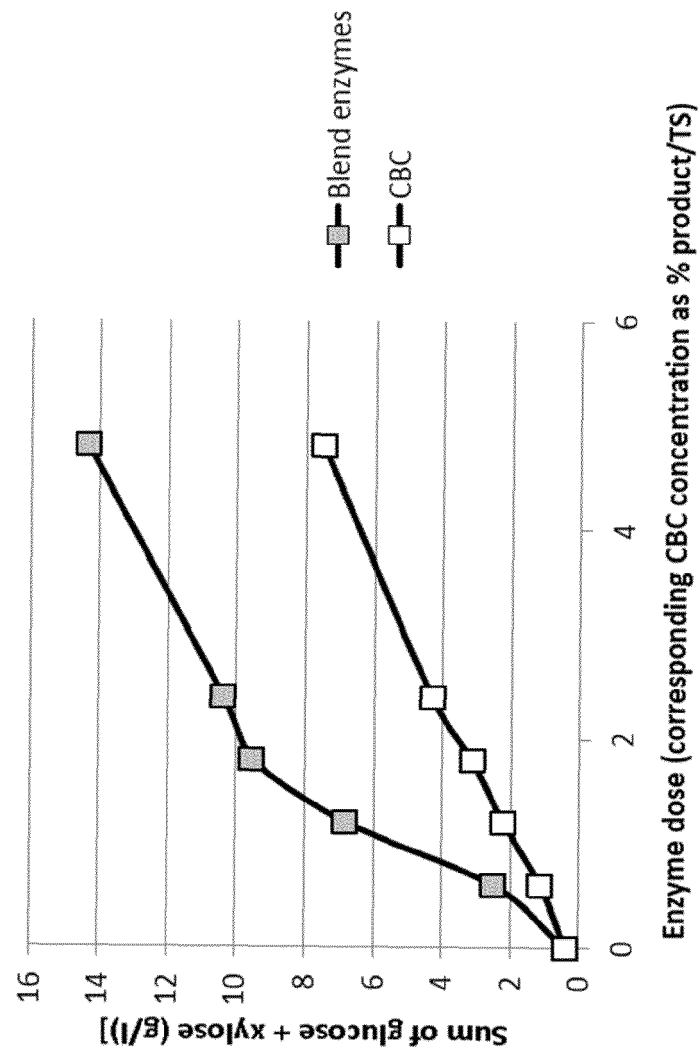


FIG 3

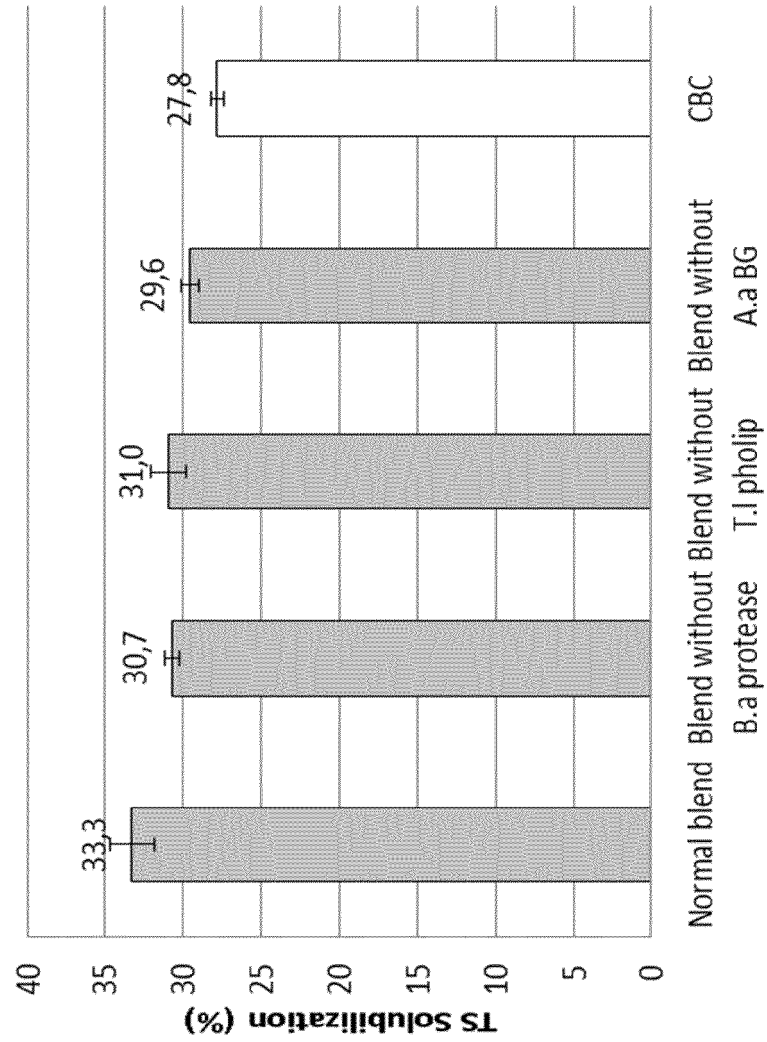


FIG 4

Mix	Enzyme dose (%/TS)	Glucose	Xylose	Lactic acid	Sum	Glucose std. dev.	Xylose std. dev.	Lactic acid std. dev.	Sum std. Dev.
No enzyme	0	0.3	0.1	5.8	6.2	0.0	0.0	0.3	0.4
Blend enzymes 25%	0.6	1.4	1.1	10.7	13.2	0.8	0.3	0.9	0.2
Blend enzymes 50%	1.2	4.9	1.9	10.0	16.8	0.4	0.1	0.2	0.4
Blend enzymes 75%	1.8	7.0	2.6	10.4	19.9	0.4	0.1	0.5	0.8
Blend enzymes 100%	2.4	7.9	2.5	9.9	20.3	0.5	0.2	0.4	0.9
Blend enzymes 200%	4.8	11.0	3.3	9.2	23.6	1.0	0.1	0.4	0.8
CBC 25%	0.6	0.4	0.7	11.1	12.3	0.0	0.3	0.2	0.5
CBC 50%	1.2	0.6	1.6	12.7	14.9	0.1	0.1	0.7	0.6
CBC 75%	1.8	1.0	2.1	13.8	17.0	0.1	0.1	0.4	0.5
CBC 100%	2.4	1.9	2.4	13.4	17.7	1.4	0.1	0.7	0.7
CBC 200%	4.8	4.4	3.1	14.6	22.1	0.5	0.2	0.3	0.8

FIG 5

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